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STUDY ON THE MECHANISM OF PICROTOXIN-INDUCED OVULATION IN THE RABBIT[1]

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PICROTOXIN INDUCED OVULATION

THE INDUCTION of ovulation by Marshall and Verney (1936) through electrical stimulation of the hypothalamic region was a major step forward in the study of the mechanism of ovulation. They reported (1938, 1939) that picrotoxin in convulsant dose (1 mg./kg. of body weight) caused ovulation in 6 of 21 rabbits tested. With few exceptions all the animals showed signs of follicle growth or hemorrhagic follicles but ovulation occurred in only 22 per cent of the animals tested. Usually the normal number of 3 to 5 ovulating follicles was found but the latent period between picrotoxin stimulation and ovulation was from 24 to 48 hours as compared to the normal period of 10 hours between copulation and ovulation. A smaller dose of picrotoxin (0.8 mg./kg.) in the presence of atropine caused violent convulsions, but none of the rabbits so treated ovulated. Since this dose of picrotoxin did not produce ovulation in the absence of atropine, it was felt that the failure was due to the smaller dose of picrotoxin. They interpreted this as an indication that no correlation existed between the convulsant activity of picrotoxin and its effect on the ovaries. Further, Makepeace (1938) found that atropine did not prevent ovulation postcoitum, but he did not use isolated rabbits in this study. Other convulsants were employed—strychnine, coriamyrtin—and general excitants—apomorphine, beta tetrahydronaphthylamine and ergometrine—but proved equally inactive. Marshall, Verney and Vogt (1939) inferred that the "absence of response might be due to failure in finding the optimal conditions for their action."

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Copper salts administered intravenously were found more predictable in the production of ovulation in rabbits by Fevold, Hisaw and Greep (1936). They felt that these salts as well as picrotoxin probably caused ovulation by augmenting the effect of the gonad stimulating hormones in the blood to the level essential for ovulation. On the other hand, Bischoff (1938) felt that the injection of so toxic a substance as copper caused ovulation through nervous stimulation. The problem was resolved by Brooks, Beadenkopf and Bojar (1940) who sectioned the pituitary stalk in rabbits and found that 10–15 mg. of copper acetate—a dose universally effective in producing ovulation in the intact rabbits which survived the injection—failed to produce ovulation. When the hypophyseal stalk was cut in 11 animals, injections of picrotoxin also failed to result in ovulation. Metrazol was similar in its ovulatory action but less effective than picrotoxin. Convulsant doses of insulin, benzedrine and ephedrine chloride all failed to induce ovulation. They too felt that conditions must be favorable for ovulation to occur before picrotoxin and metrazol could be effective, but were unable to explain why such dissimilar substances should cause ovulation to occur in the rabbit when various other excitants and convulsants were inactive in this respect.

An excellent review of the pharmacological activity of picrotoxin was published by Dille (1938, 1939) and will not be discussed here. The specific antagonistic action of barbiturates for picrotoxin was described by Swanson (1932) and has been reviewed by Tatum (1939). That barbiturates themselves do not interfere with ovulation has been adequately demonstrated by Brooks and his colleagues (1940). This protective action of barbiturates makes it possible to use picrotoxin in several times the effective ovulating dose without sacrifice of the animals. It could then be established whether the effect of picrotoxin on ovulation is directly related to its convulsant properties or to the quantity of picrotoxin injected into the rabbit.

The convulsant action of picrotoxin was early studied by Pagniez (1929). It was found to have a depressant effect upon the rhythmic activity of leech muscle (Gessner, 1932), as well as a vasoconstrictor action not antagonized by ergotamine (Gessner, 1928). Miller and Spiegel (1939) decorticated rats and administered picrotoxin to establish the part played by the cerebral cortex but found it just as effective a convulsant in the decorticate animals. Schriever and Perschmann (1936) localized the seat of action to the midbrain which is significant in that the hypophysis is found to be an essential link in the catenary system whereby picrotoxin causes ovulation.

Further, the fact that rabbits which have just littered are highly susceptible to mating makes it possible to obtain animals "primed" for ovulation. The ovary of the postpartum rabbit was found "primed" for ovulation by Friedman (1932) and responded to the injection of one minimal effective dose of extract of pregnancy urine. It was felt

that the use of puerperal animals would make it possible to satisfy to some extent the "favorable conditions" for ovulation that both Marshall and Brooks and their colleagues have stressed as essential in the ovulatory activity of picrotoxin.

METHOD

Mature but virgin female rabbits, healthy and well fed, were isolated for at least 20 days before use in order to control the occurrence of single hemorrhagic follicles frequently found in such animals. Phenobarbital was used in 10 per cent aqueous solution and the picrotoxin was used in 20 per cent alcoholic solution. Laparotomy was performed under drop ether anaesthesia with phenobarbital used as a basal anaesthetic in some animals and oophorectomy was performed in all suspicious cases and in most of the others. All laparotomies were performed 48–72 hours after the injection of the picrotoxin. The animals injected after parturition received picrotoxin intravenously within 12 hours of the time the litters were dropped. Tissues were fixed in 10 per cent formalin and stained with hematoxylin-eosin. In every case the diagnosis of ovulation was established by microscopical study of the tissues. Hemorrhagic follicles were not recorded as proof of ovulation. The importance of identifying ovulation by means of microscopical examination of the ovary or by actual recovery of the ova from the tubes has been stressed by Lewis and Wright (1935).

RESULTS

As a control experiment, the work of Marshall, Verney, and Vogt (1939) was repeated using the dosage and technique which they found

TABLE 1. PICROTOXIN INJECTIONS IN ISOLATED VIRGIN DOES

Picrotoxin mg./kg. i.v.	Hyperactivity + and Convulsions + +	Ovulation
1.0	+ +	−
1.0	+	−
1.0	+ +	+
1.0	+ +	+
1.0	+	−
1.0	+	−

successful in causing ovulation in the rabbit. Accordingly, a series of 6 rabbits received 1.0 mg./kg. of picrotoxin intravenously (sc. Table 1) but in spite of the production of convulsions in half of them, ovulation was found at laparotomy in but 2. This failure to induce ovulation in more of the normal isolated does at levels of picrotoxin found adequate in some rabbits under identical conditions serves but to emphasize the uncertain chain of events that is apparently essential for the occurrence of picrotoxin-induced ovulation in the rabbit.

In the next series the rabbits used had dropped their litters within 12 hours of the time picrotoxin was administered in order to minimize as much as is possible by this means variation in susceptibility for

ovulation in the host. Twenty-four rabbits were employed with results shown in Table 2. The first 10 animals received 1.0 mg./kg. of picrotoxin intravenously and none ovulated in spite of severe convulsions which occurred in half of them. The remaining 5 rabbits in this group showed great excitability. The dose was then raised to 1.5 mg./kg. of body weight injected intravenously, a dose well above the level found capable of producing ovulation by Marshall, Verney and Vogt. All of these rabbits developed severe convulsions and 2 of the 14 so treated expired in their course. In 2 rabbits the presence of ovulation was demonstrated microscopically. Thus, ovulation occurred in but 12.5 per cent of the rabbits surviving the convulsions. It is significant that in spite of a larger dose of drug, in spite of the

TABLE 2. PICROTOXIN INJECTIONS IN "PRIMED" RABBITS 12 HOURS POST-PARTURITION

Picrotoxin mg./kg. i.v.	Hyperactivity + and Convulsions + +	Ovulation
1.0	+ +	expired
1.0	+	−
1.0	+ +	−
1.0	+	−
1.0	+ +	−
1.0	+	−
1.0	+ +	−
1.0	+ +	−
1.0	+	−
1.0	+	−
1.5	+ +	−
1.5	+ +	+
1.5	+ +	−
1.5	+ +	−
1.5	+ +	−
1.5	+ +	expired
1.5	+ +	−
1.5	+ +	−
1.5	+ +	+
1.5	+ +	expired
1.5	+ +	−
1.5	+ +	−
1.5	+ +	−
1.5	+ +	−

presence of convulsions, and in spite of rabbits "primed" for ovulation in the postparturitional state, such a small number were found to ovulate.

In the third group of barbiturate protected animals, the picrotoxin was administered intravenously in doses much larger than those administered by Marshall, Verney, and Vogt. Even with the relatively small dose administered intravenously 20 per cent of their unprotected rabbits expired during the convulsions. The minimal lethal dose of picrotoxin for rabbits has been established by Swanson and Chen (1936) as 2.5 mg./kg. when administered intravenously and only slightly higher when given subcutaneously. The minimal lethal dose for phenobarbital in rabbits is in the neighborhood of 150

mg./kg. From Table 3 it can be seen that from 2 to 6 times the dose of picrotoxin used by Marshall or by Brooks and their colleagues was administered to the rabbits protected against such a lethal dose by phenobarbital without evidence of ovulation at laparotomy. Convulsions occurred only in the 3 animals receiving the largest dose of picrotoxin, 2 of which expired during convulsions. In the one rabbit surviving the convulsions no evidence of ovulation was found at laparotomy. The significance of this series is that picrotoxin in a dose 2 to 6 times that found capable of inducing ovulation was completely ineffective here.

TABLE 3. PICROTOXIN INJECTIONS IN VIRGIN ISOLATED RABBITS PROTECTED BY PHENOBARBITAL

Picrotoxin mg./kg. i.v.	Phenobarbital mg./kg. Subcutaneously	Hyperactivity + and Convulsions + +	Ovulation
2.0	100.0	+	−
2.0	100.0	+	−
3.0	120.0	+	−
6.0	120.0	+	−
6.0	160.0	+ +	−
9.0	120.0	+ +	expired
12.5	66.5	+ +	expired

DISCUSSION

The evolution of the process whereby ovulation occurs is still in the period of development but the last 20 years have seen important advances made. With the use of both normal and hypophysectomized rabbits a definite relationship between ovulation and the anterior lobe of the pituitary was demonstrated (Bellerby, 1934). The early study of Fee and Parkes (1929) on the rabbit showed that hypophysectomy within one hour of copulation inhibits ovulation, but later than one hour does not. Friedgood and Pincus (1935) showed that the anterior hypophysis was apparently directly responsible for the initial maturation of ovarian follicles through its gonadotropic properties. That the sympathetic nervous system is involved is accepted, but the fact that ovulation could occur in rabbits with the thoracolumbo-sacral sympathetic system (Hinsey and Markee, 1932) as well as the cervical sympathetics interrupted (Haterius, 1934) places this system early in the series of events which lead eventually to ovulation. It is quite evident that the anterior pituitary lobe is a direct effector by a humoral route through which ovulation occurs in the rabbit.

In this study the ability of picrotoxin to induce ovulation in the rabbit was studied, and several significant facts elucidated. In one series picrotoxin in a dose several times that found adequate to induce ovulation in the rabbit was administered to does protected by phenobarbital against the lethal quantities injected. Ovulation resulted in none of the rabbits including one which survived severe con-

vulsions following a dose 6 times that found effective and more than twice the minimal lethal dose. The fact that ovulation did not obtain although there can be no question of the fact that the picrotoxin was physiologically effective is significant. The barbiturate was exonerated in antagonistic effect by the report of Brooks, Beadenkopf and Bojar (1940). This suggests that the ovulatory activity of picrotoxin is not directly related to the quantity of drug in the body. It is also noteworthy that frequently in this series of rabbits a moderately severe hyperemia of the uterus and tubes was found. Although the number of animals used is admittedly small, it is demonstrated that larger doses of picrotoxin are certainly no more effective than those one-half to one-sixth as great found irregularly capable of producing ovulation.

A control group of animals received a dose of picrotoxin reported to be effective in causing ovulation to occur in less than one third of the rabbits tested and one third of these were found to ovulate. That it can do so at times is undeniable, but it is apparently difficult to predict the ovulatory effect of picrotoxin. That convulsions in themselves are not sufficient to induce ovulation in the rabbit is apparent from the numerous failures following convulsions caused by insulin, strychnine, coramine and electrical stimulation. In addition, although ovulation following picrotoxin injection occurs in convulsant doses, many rabbits which experienced convulsions did not ovulate.

In a third series of rabbits injected after parturition which were in an optimal "primed" condition for ovulation but 2 of 21 rabbits (10 per cent) surviving a dose considered effective ovulated. This level of successful activity is lower than that reported by Marshall and by Brooks and their colleagues as well as in the control series of rabbits here reported. The failure to induce ovulation more frequently in these "primed" rabbits was somewhat unexpected, and serves to make even more obscure the "favorable conditions" under which picrotoxin can produce ovulation. "Priming" was also attempted by Brooks, Beadenkopf and Bojar by injection of sub-ovulating doses of pituitary follicle-stimulating hormone (FSH). This did not increase the frequency of ovulation following picrotoxin injections in the rabbit.

In spite of the small numbers of animals employed in these experiments it can be stated that picrotoxin induced ovulation in the rabbit occurs unpredictably at convulsant levels in approximately one-ninth of the animals tested. The fact that 2 to 6 times the effective dose did not induce ovulation suggests that it is not the quantity of the drug alone which determines its ovulatory activity. However, it is just possible that, in spite of evidence to the contrary, quantities of barbiturates sufficient to protect the rabbits against levels of picrotoxin greater than the lethal dose may interfere with its ovulatory activity. Finally, it is apparent that lack of susceptibility of the host

to ovulation, as determined by the post-parturitional "primed" state of the rabbit for ovulation, is in itself not a final determinant of picrotoxin-induced ovulation in the rabbit.

SUMMARY

The induction of ovulation in the isolated rabbit doe was found to occur in a total of 4 out of 27 animals (15 per cent) surviving the administration of picrotoxin in reported adequate dose.

Two to 6 times the effective ovulatory dose of picrotoxin fails to induce ovulation in rabbits protected against the lethal quantity of picrotoxin by phenobarbital.

The frequency of successful induction of ovulation with picrotoxin is not increased by augmenting the susceptibility of the host by means of the ovulation-primed state following parturition.

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A NEURAL FACTOR IN THE MECHANISM BY WHICH ESTROGEN INDUCES THE RELEASE OF LUTEINIZING HORMONE IN THE RAT[1]

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INTRODUCTION

In polyestrous animals such as the rat, mouse, and guinea pig, which ovulate spontaneously, estrogen stimulates the release of luteinizing hormone from the hypophysis (Fevold, 1939; Hellbaum and Greep, 1946; Hisaw, 1947; Everett, 1947, and Bradbury, 1947). The liberated LH[2] proceeds to induce preovulatory swelling, ovulation, formation of new corpora lutea and, in pregnant or pseudopregnant rats, cholesterinization of existing active corpora lutea (Everett, 1947). The spontaneously ovulating species do not require an overt neural stimulus, such as is presumably initiated by copulation, for the release of the gonadotrophic hormone. Nor is it established that coitus hastens ovulation in these animals. In this respect they differ from such species as the rabbit, cat and ferret, in which the release of an ovulating surge of LH normally occurs only after copulation. Conversely, estrogen alone will not stimulate discharge of LH in the animals of the latter type (Bachman, 1936; Dury and Bradbury, 1943).

There is considerable evidence that, in the spontaneously ovulating group, estrogen acts directly upon the hypophyseal cells with no intermediation whatever by the nervous system. This evidence derives from three sources: (1) Hypophyseal grafts, which are of necessity removed from normal neural relationships, have been reported to maintain the integrity of sex function in hypophysectomized rats (Greep, 1936; May, 1937). (2) Section of the pituitary stalk, which severs the nerve fibers entering the hypophysis, has been asserted to be consistent with normal sex function in the guinea pig (Dempsey, 1939; Dey, 1943), in the rat (Dempsey and Uotila, 1940; Dempsey and Searles, 1943) and in the dog (Brekenridge and Keller, 1948).

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[2] LH is used in the conventional sense (cf. Hisaw, 1947) although its effects here may largely express quantitative rather than qualitative differences in gonadotrophic secretion.

(3) Cytological changes, induced by massive doses of estrogen, occur in hypophyseal grafts (Desclin and Gregoire, 1936; Martins, 1936, a; Hohlweg, 1936; Phelps, Ellison and Burch, 1939) and in stalk-sectioned pituitaries (Westman and Jacobsohn, 1938, a; Uotila, 1940) as readily as in non-operated hypophyses (Hohlweg, 1934; Severinghaus, 1939; Baker and Everett, 1947).

A nervous element in gonadotrophic control is suggested by evidence from the same sources: (1) More or less impairment of sex function has been reported in guinea pigs (Schweizer, Charipper and Haterius, 1937) and in rats (Westman and Jacobsohn, 1940) which have been hypophysectomized and implanted with pituitary grafts. (2) Mild to severe symptoms of gonadotrophic upset have been claimed as sequelae of section of the pituitary stalk (Richter, 1933; Westman and Jacobsohn, 1937, 1938; Herold, 1939; Herold and Effkemann, 1938; Westman, Jacobsohn and Hillarp, 1943; Brolin, 1945). (3) Certain cytological changes in the hypophysis appear to require a neural stimulus. Castration cells which ordinarily form in response to gonadectomy do not appear in grafted hypophyses (Hohlweg and Junkmann, 1932; Desclin and Gregoire, 1936; Martins, 1936, b; Westman and Jacobsohn, 1940) nor in pituitaries whose stalks have been severed (Westman and Jacobsohn, 1938, a).

The investigators whose results are so diametrically opposite have criticized each other's techniques. For instance Uotila (1940) suggests that Westman and Jacobsohn's stalk-section operation damaged the anterior hypophysis, whereas Westman, Jacobsohn and Hillarp (1943) point out that Uotila's stalk-sections were incomplete. Obviously a procedure which would cause no structural damage to either nerves or blood vessels and yet would prevent the effects of nervous activity from reaching the hypophysis would be a highly desirable method to resolve the controversy. Such a technique has recently proved effective in the rabbit in preventing the neurogenic stimuli attending copulation from releasing LH (Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Townsend, 1949). The technique consists of injecting adrenolytic or anti-cholinergic agents into the animal at the appropriate time. The rabbit evidence indicates that the neurogenic stimulus for the release of LH includes both cholinergic and adrenergic components, the former initiating the latter (Sawyer, Markee and Townsend, 1949). That the blocking drugs, Dibenamine and atropine, inhibit ovulation by preventing the stimulus for LH release from reaching the hypophysis, rather than by interfering with the liberation of the hormone from the gland itself, is indicated by this fact: delaying injection less than a minute post coitum permits ovulation to proceed in an unimpaired manner although at least an hour is consumed in the discharge of LH (Fee and Parkes, 1929).

The blocking technique has therefore been applied to the problem of whether estrogen stimulation of the hypophysis, leading to the release of LH in a spontaneously ovulating species, represents a direct

effect of estrogen on hypophyseal cells or an indirect stimulation via the nervous system. The study takes advantage of another new technique, one which readily reflects the release of LH, and to a certain extent registers its quantitative aspects and time relationships, in response to estrogen in the rat (Everett, 1947). If female rats are injected with estrogen on the fourth day of pregnancy, they regularly ovulate on the second night thereafter and a rich deposition of cholesterol occurs in the corpora lutea at about the same time. LH injected on the fifth day of pregnancy causes both ovulation and cholesterol storage within 18 hours even if the hypophysis is removed at the time of injection.

In the present study we have administered blocking agents in conjunction both with estrogen and with LH. The results to be presented below indicate strongly that the estrogen stimulus for the release of LH is exerted primarily upon the nervous system and only indirectly on the hypophysis itself. The neurogenic stimulus, initiated or facilitated by estrogen, contains both cholinergic and adrenergic components as in the rabbit. Neurogenic stimuli effective in releasing sufficient LH to induce ovulation are not initiated, under the conditions of the experiment, until at least 20 hours after the administration of estrogen. Part of the results of the present work have already been reported in abstract form (Sawyer, Everett, and Markee, 1948; Everett, Sawyer and Markee, 1948).

MATERIALS AND METHODS

Sixty sexually mature female albino rats of the inbred Vanderbilt strain (a derivative of the Osborne-Mendel colony) were used in this study. Each was mated during proestrus, as determined by daily vaginal smears, with a known fertile male. If sperm were present in the smear the following morning, pregnancy was dated with that morning as zero time.

Estradiol benzoate was administered in sesame oil by subcutaneous injections. The standard dose, at a concentration of 0.33 mg./ml., was 50 μg. (0.15 ml.). Two samples of purified sheep hypophyseal LH[3] were used (*P 118* and *15HM3*).Doses of 3 mg. of either material in aqueous solution (10 mg./ml.) were known to be fully effective in ovulating pregnant rats and producing cholesterinization of their corpora lutea within 18 hours.

Solutions of Dibenamine hydrochloride and atropine sulfate (USP XIII, Mallinckrodt) were prepared from the salts just prior to injection. Dibenamine was first dissolved in 95% alcohol at a concentration of 100 mg./ml. and this solution was diluted to a final concentration of 10 mg./ml. with unbuffered Ringer-Locke solution. With the use of a tuberculin syringe and a

[3] These preparations of LH were generously contributed by Dr. R. K. Meyer, University of Wisconsin. They were assayed in his laboratory for both FSH and LH activity. In *P 118* no FSH was detectable in 3.3 mg. (0.5 gm. equivalent of the whole dried gland), yet the same quantity augmented ovarian stimulation by a standard FSH preparation (average ovarian weight in LH and FSH: 60 mg., contrasted with 45 mg. produced by FSH alone). In *15HM3* very slight FSH activity was found in 5 mg. (0.5 gm. equivalent), while the same quantity with FSH gave marked augmentation (average ovarian weight: 115 mg.).

27 gauge needle the Dibenamine solution was injected very slowly (about 0.5 ml./minute) into the small saphenous vein of the unanaesthetized rat. The injection was performed slowly because the amount of the drug tolerated on slow administration is much greater than on rapid injection (Nickerson and Goodman, 1947). The foot and leg were prepared by swabbing with 70% alcohol and shaving off the hair, and the vein was usually entered below the ankle. The injection technique requires two people, one to hold the rat, previously wrapped in a towel, and the other to perform the injection. The site is an excellent one for highly effective intravenous injections because the outline of the vein is sharply demarcated, and the needle may be held in the vein by the operator's hand which is clasping the rat's foot.

Preliminary experiments to determine the optimal dosage of Dibenamine employed 15 adult male rats. They were injected with doses ranging from 20 to 40 mg. per kg. For reasons to be presented below, 30 mg./kg. was adopted as the standard blocking dose although, as we shall see, it is not completely effective in blocking estrogen-induced liberation of LH.

Atropine sulfate was dissolved directly in Ringer-Locke solution. Preliminary attempts to find an intravenous blocking dose revealed that rats would tolerate a dose of 50 mg./kg. while 60 mg./kg. was lethal to one and almost fatal to 2 others out of 4. The intravenous injection of 50 mg./kg. atropine, however, failed to block estrogen-induced ovulation, probably because its effect was too short-lived. Therefore, the subcutaneous route was subsequently employed. The solution was made up as 70 mg./ml.; at this concentration it required only a simple calculation to administer the effective subcutaneous dose, 700 mg./kg. (Holck, 1942)—one ml. was injected per 100 mg. of body weight.

In a typical experiment Dibenamine was injected intravenously at 11 A.M. on the fourth day of pregnancy, and one hour later estradiol benzoate was injected subcutaneously. In another series of experiments, estrogen was administered at noon on the fourth day of pregnancy and Dibenamine at 8 A.M. on the fifth day. Atropine was injected subcutaneously, always at about 8 A.M. on the fifth day of pregnancy. LH was injected at 11 A.M. or noon on the fifth day. The animals were usually killed with illuminating gas and autopsied on the morning (occasionally early afternoon) of the sixth day. The ovaries were examined in physiological saline under a dissecting microscope for external evidences of stimulation by LH: hypertrophy, hyperemia or rupture of the largest set of follicles and a shift in the relative lipid content of interstitial tissue and corpora lutea of pregnancy. Tubal ova were visualized by removing the ampullar end of the oviduct and flattening it in saline with a cover slip on a microscope slide.

One or 2 corpora lutea were removed for fixation in 10% formol and the remainder of the ovary was fixed in Zenker's solution. The Zenker-fixed material was embedded in paraffin, sectioned at 10μ, or occasionally at 15μ, and stained with Harris' hematoxylin and a modification of Mallory's triacid stain (Everett, 1943). The formalin-fixed pieces were sectioned on the freezing microtome at 30μ and were studied for cholesterol content by the Schultz technique (Everett 1945, 1947).

RESULTS

The optimal dose of Dibenamine. In preliminary tests of survival at various Dibenamine dosage levels, employing 15 male rats, the

following results were obtained (the dosage is followed by the proportion which survived): 20 mg./kg., 1/1; 25 mg./kg., 5/5; 30 mg./kg., 4/6; 35 mg./kg., 1/2; and 40 mg./kg., 0/1. The animal which received the 40 mg./kg. dose and one of those which was injected with 30 mg./kg. died almost immediately; the others which failed to survive succumbed during the first night. The rest lost from 5 to 15% of their body weight during the next 2 or 3 days, the loss being attributable at least in part to a temporary loss of appetite.

Inasmuch as 25 mg./kg. was the highest dose which all the rats survived, this dosage was injected in the first attempts to block estrogen-induced ovulation. Of 5 female rats receiving this dose of Dibenamine prior to estrogen administration (5 μg. estradiol benzoate) on the fourth day of pregnancy, 3 were found at autopsy to have ovulated. The 5 μg. injection of estradiol benzoate was not completely effective in inducing ovulation: when this dose was administered in the absence of Dibenamine, only 2/4 rats ovulated. However, assuming 5 μg. estrogen to be a completely adequate stimulus, 25 mg./kg. of Dibenamine would be only 40% (2/5) effective in blocking estrogen-induced ovulation. Therefore a dose of 30 mg./kg. of Dibenamine was adopted for further experiments which assayed to block the estrogen induced liberation of LH.

Effects of Dibenamine and atropine not directly concerned with gonadotrophic function. No attempt was made to study in detail the pharmacological actions of Dibenamine and atropine. We were, however, impressed by the constancy of certain sequelae of drug administration to the female rats in attempted-blocking experiments. Some of these observed responses will be reported here.

Within a few seconds after an intravenous injection of Dibenamine (10 mg./ml.) was begun, a predictable jerk of skeletal musculature would all but dislodge the needle. From that instant until the termination of injection (the dose of 30 mg./kg. required 0.60 ml. for a 200 gm. rat, and the time consumed was more than a minute) the musculature remained rigid or trembled. Within the next 2 or 3 minutes, following release of the rat to its cage and continuing for 5 to 10 minutes, spontaneous contractions of the extensor trunk musculature would pull the rat to an upright posture on its hind legs, from which position it would fall backwards or sidewise. The sequence would be repeated several times, after which the rat would remain prone and quiescent for some hours. Even the following day the rat usually was lethargic and often felt cold to touch. It was observed not to feed during the first several hours after injection and undoubtedly lost weight, as did the males in preliminary experiments. The dose of 30 mg./kg. of Dibenamine was somewhat better tolerated by female rats in blocking experiments than by the males in preliminary injections, perhaps due to more careful, slower administration to the females. Only 3 females of 45, which received Dibenamine in the current series, expired before the intended hour at which each experiment was to be terminated.

Certain observed reactions to Dibenamine might be classified as cholinergic phenomena. Within a few minutes after injection there was usually profuse salivation, occasionally so copious that it interfered with breathing. Within an hour after injection there was often evidence of chromodacryorrhea, porphyrin secretion by the Harderian glands, appearing as "bloody" tears. The porphyrin nature of the secretion was ascertained in the present experiments by its red fluorescence in ultraviolet light. Chromodacryorrhea is a cholinergic response and has been used as a test for the presence of acetylcholine (Tashiro et al., 1940). The pupillary constriction, however, which was so constant in the Dibenamine-treated rabbit (Sawyer et al., 1947), was only occasionally observed in the rat. Another fairly common occurrence was the excretion, within the first hour after injection, of bloody urine. The pigment in the urine was found, on spectroscopic analysis, to be actually oxyhemoglobin. The presence of hemoglobin in the urine, in contrast with secreted porphyrin in the tears, is indicative of extensive destruction of erythrocytes by Dibenamine.

A quite constant effect of Dibenamine in conjunction with estrogen (but not with LH) was the subcutaneous deposition of a mucoid material in the thigh, inguinal region and sometimes the whole abdomen on the side of the Dibenamine injection site. The mucoid was readily located even without making an incision because it separated the skin and the underlying muscle in such a way that the skin appeared translucent. As will be mentioned below there appeared to be a positive correlation between the abundance of the mucoid and the degree of blockage of estrogen-induced release of LH.

Subcutaneous injection of atropine sulfate (700 mg./kg.) was followed within a minute or two by maximal dilatation of the pupil. Before autopsy the external abdominal wall was observed to be wet with urine and this incontinence was related at autopsy to a maximally dilated, atonic bladder. The skin and connective tissue at the site of injection were very hyperemic and the underlying muscular wall was rigidly contracted.

Injection of estrogen alone on the fourth day of pregnancy. Subcutaneous injection of 50 μg. estradiol benzoate into 8 female rats on the fourth day of pregnancy resulted in ovulation and cholesterol storage in all 8 as observed at autopsy on the sixth day (figure 1, A). All of the pregnancies were interrupted: none of the uteri were gravid on the sixth day. Tubal ova were visualized and rupture points of new corpora lutea were observed in each case. The corpora lutea of pregnancy all appeared fatty and all gave strongly positive (++) Schultz tests. These observations confirm the findings of Everett (1947). In figure 1, A (as also in 1, C, and 2, D) the + Schultz reaction shown in the fifth-day corpus luteum is based directly on the earlier work; none of the 8 estrogen-treated females in the present study was autopsied before the sixth day.

Injection of both Dibenamine and estrogen on the fourth day of pregnancy. The results of injecting Dibenamine into 12 female rats one

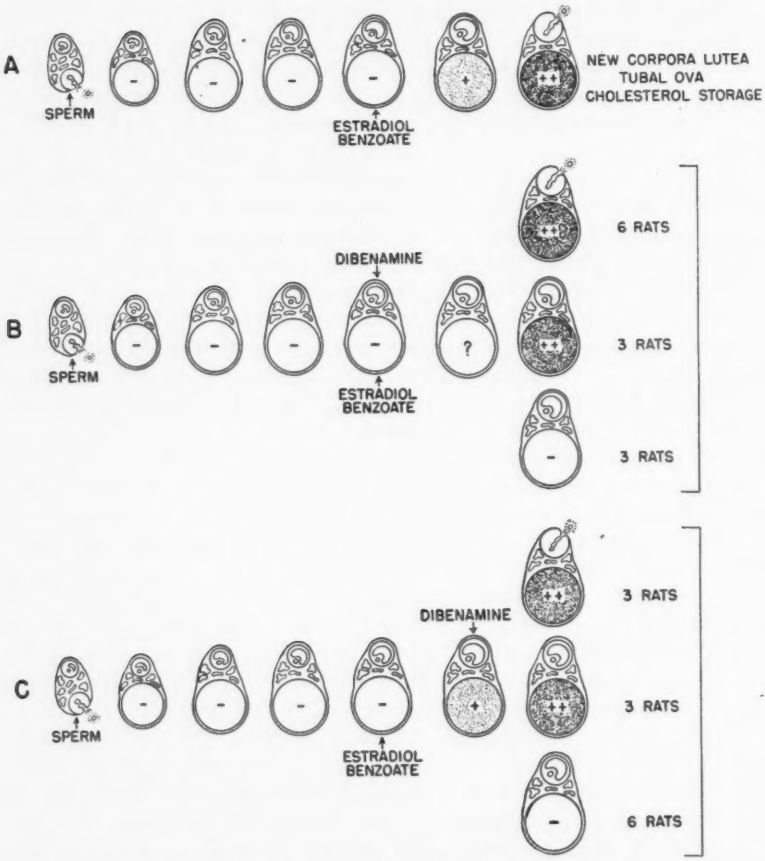

FIG. 1. Diagrammatic illustrations of experiments on the effects of estrogen and Dibenamine administration to the pregnant rat. Each horizontal group depicts the daily growth of a follicle and a corpus luteum of pregnancy from the day on which sperm were found in the vaginal smear (zero time). The symbols minus (−) and plus (+) represent degrees of luteal cholesterinization as determined by the Schultz test.

A. Estrogen, injected on the fourth day of pregnancy, induced LH release as evidenced by ovulation and storage of cholesterol in the corpus luteum of pregnancy within 48 hours. A slight cholesterinization was evident at 24 hours (Everett, 1947).

B. Dibenamine, injected just before estrogen on the fourth day, blocked estrogen-induced ovulation in 6/12 rats and blocked cholesterol storage in 3/12 rats.

C. Dibenamine, injected 20 hours after estrogen, blocked estrogen-induced ovulation in 9/12 rats and blocked cholesterinization in 6/12 rats.

hour prior to the administration of estrogen on the fourth day of pregnancy are summarized in figure 1, B. Nine rats of this series revealed strongly Schultz-positive corpora lutea and 6 of these 9 ovulated. The follicles in the 6 rats which failed to ovulate showed no evidence of stimulation by LH; they contained ova with resting nuclei and there was no indication of secondary follicular liquor.

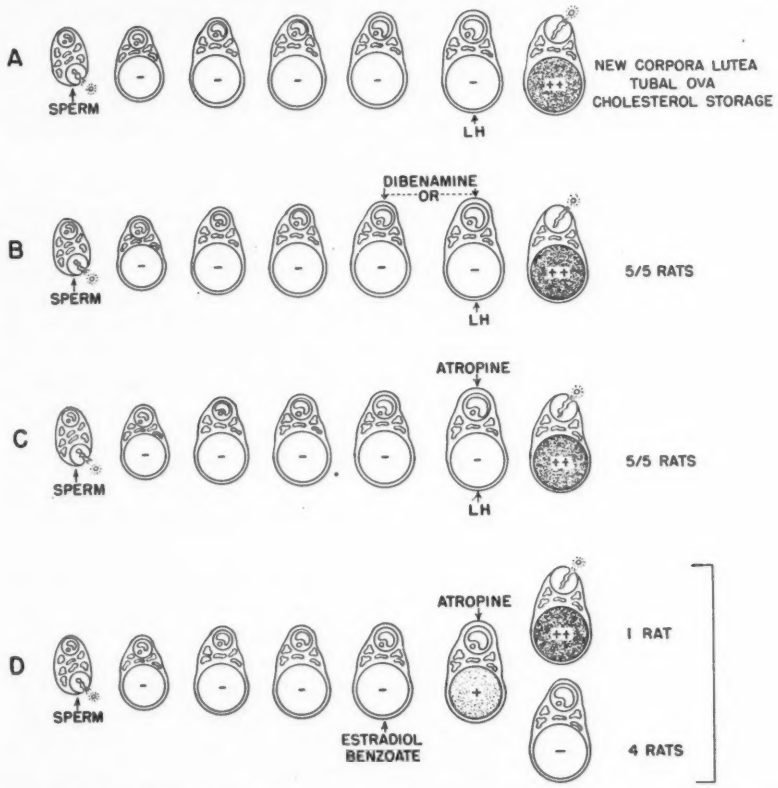

FIG. 2. A. LH induced ovulation and luteal cholesterol storage in pregnant rats within 24 hours after injection (Everett, 1947).

B. Dibenamine, injected 24 hours before or simultaneously with LH, failed to block the effects of LH on ovulation and cholesterol storage.

C. Atropine also failed to inhibit the effects of LH administration.

D. Atropine, injected subcutaneously as much as 20 hours after estradiol benzoate, quite effectively blocked the estrogen-induced release of LH as evidenced by failure of ovulation and cholesterinization in 4/5 rats.

Injection of estrogen on the fourth day and Dibenamine on the fifth day of pregnancy. The results of injecting estrogen at noon on the fourth day of pregnancy and Dibenamine 20 hours later are summarized in figure 1, C. Six rats stored cholesterol in their corpora lutea and 3 of these ovulated. Sections of the ovaries of the 9 rats which failed to ovulate revealed no preovulatory swelling—all of the oocytes had resting nuclei.

As was mentioned above, there was considerable positive correlation between the amount of subcutaneous mucoid deposited and the blocking effectiveness of Dibenamine. Twelve animals, among the 24 Dibenamine-treated rats, contained large amounts of mucoid. Only

2 of the 12 ovulated. On the other hand, 9 rats had little or no mucoid, and 6 of these ovulated; the other 3 had strongly Schultz-positive corpora lutea. The remaining 3 (out of 24) had a mucoid deposit of intermediate size; one was completely blocked, one stored cholesterol in corpora lutea without ovulating, and the third ovulated and had Schultz-positive (++ to +++) corpora lutea.

Injection of LH on the fifth day and Dibenamine on the fourth or fifth day of pregnancy. Figure 2, A graphically summarizes the results of injecting LH on the fifth day of pregnancy: ovulation and cholesterol storage in the corpora lutea of pregnancy at autopsy on the sixth day (Everett, 1947). Of 5 female rats, in the present experiments, which received LH on the fifth day, 3 received Dibenamine at 11 A. M. on the fourth day and 2 at 8 A. M. on the fifth day. As illustrated in figure 2, B, all 5 ovulated and had strongly Schultz-positive corpora lutea. The rats differed from many of the estrogen-Dibenamine-treated animals in this respect—none revealed any abdominal mucoid whatever. Whether the mucoid requires a high estrogen level for its manifestation or whether conditions attending ovulation induce its dissolution cannot be determined from the present data.

Injection of atropine and LH on the fifth day of pregnancy. Just as Dibenamine fails to block the effects of LH administration so does atropine. Figure 2, C summarizes the results of injecting 700 mg./kg. atropine sulfate subcutaneously at 8 A.M. and LH at 11 A.M. on the fifth day of pregnancy. At autopsy all had gravid uteri and dilated ampullae with visible ova. The ovaries revealed new corpora lutea with rupture points, a somewhat dull and watery interstitial tissue and fatty corpora lutea of pregnancy which gave a ++ to +++ Schultz reaction.

Injection of estrogen on the fourth day and atropine on the fifth day of pregnancy. Five pregnant rats were treated with atropine 20 hours after estrogen as illustrated in figure 2, D. Of these, 4 showed no signs whatever of stimulation by LH. The ovaries contained fatty interstitial tissue, pallid corpora lutea of pregnancy and clear, non-hyperemic follicles about 0.7 mm. in diameter. In sections these follicles were observed to contain ova with resting nuclei only. The fifth rat revealed no tubal ova nor readily visible ruptured follicles; the only external sign of stimulation was a slightly hyperemic, less fatty interstitial tissue. In sections, however, one follicle was observed to have ruptured, and another showed preovulatory changes; furthermore the corpora gave a ++ Schultz test.

Not illustrated in the diagrams are the negative results with intravenous atropine. Three pregnant rats treated with estrogen on day 4 were injected intravenously with atropine sulfate (50 mg./kg.) at 8 A.M. on day 5. At autopsy on day 6 they all showed completed ovulation, interstitial tissues considerably depleted of lipid and fatty

corpora lutea which gave strongly positive Schultz reactions. The failure of intravenous atropine to block the discharge of LH may well be due to elimination or inactivation of the drug prior to the beginning of stimulation of the LH release mechanism.

DISCUSSION

It is a well established fact that sex hormones can in some way influence nervous activity. For instance, behavior may be considerably modified by an excess or deficiency of estrogen or testosterone (Beach, 1948). It has also long been recognized that the nervous system may be involved in the release of certain hypophyseal hormones. Luteinizing hormone in the rabbit and luteotrophic hormone in the rat are released in response to copulation or artificial neural stimulation. Furthermore, there is abundant evidence, to which reference has already been made, that estrogen stimulates or facilitates the release of luteinizing hormones in spontaneously ovulating species. To the best of our knowledge, however, our work presents the first convincing demonstration that the estrogen stimulus for the release of LH involves the intermediation of the nervous system.

Several earlier workers, notably Hohlweg and Junkman, Herold and Effkeman, and Westman and Jacobsohn, reported results which implicated the nervous system in sex-hormonal control of the hypophysis. These authors were largely concerned, however, with the production or synthesis of the gonadotrophic hormones rather than with their release. As will be pointed out below, their experiments on "release" are open to criticism.

Hohlweg and Junkman (1932) and Hohlweg (1936) observed that castration cells do not develop in hypophyses that have been removed from their normal neural relationships. They proposed a hypothetical "Sexual centrum" from which emanated the nervous impulses responsible for castration cell formation. When blood estrogen level reached a threshold value it inhibited the sex center from stimulating further production of FSH. Since, however, injected estrogen induced the same cytological changes in transplants as in intact hypophyses, they believed that estrogen's influence on LH was a direct one, independent of the nervous system. An interesting, although often overlooked, preliminary finding, mentioned by Hohlweg and Junkman (1932), is that atropine blocks the development of castration cells in the hypophyses of immature spayed rats. As was mentioned earlier, the observation that the formation of castration cells requires the integrity of the pituitary stalk, has been confirmed many times.

Herold and Effkemann (1938) reported results which purported to demonstrate that estrogen stimulates the release of LH via the nervous system. They cut the pituitary stalks in immature female rats and treated them with estrogen during the next 13 to 28 days, starting injections on the fifth to the eighth post-operative day. At autopsy

they noted the absence of luteinized ovarian follicles. Westman and Jacobsohn (1938, c), however, observed that stalk-sectioned hypophyses lose their gonadotrophic potency within 4 days. It seems probable, therefore, that Herold and Effkemann were attempting to stimulate the release of LH after the effective gonadotrophin content of the hypophysis had become depleted.

Westman and Jacobsohn (1938, b) considered neural stimuli entering along the pituitary stalk to be essential for the production but not for the release of gonadotrophic hormones. They observed that estrogen would induce luteinization of the ovaries of immature rats if injected 4 days before hypophysectomy or 2½ days before stalk section. This was interpreted to mean that the production of gonadotrophic hormone ceased when the stalk was cut but that estrogen, by a direct effect on hypophyseal cells, could liberate the LH already produced. We would suggest, rather, that the estrogen-facilitated neurogenic stimulus for the release of LH had reached the hypophysis by 2½ days and that release of the hormone involved a day and a half. In another paper Westman and Jacobsohn (1938, c) claimed to have demonstrated the stimulation of release of "gonadotrophic" hormone in adult estrous rats whose stalks had just been severed. Electrical stimulation of the vaginae, 2 to 5 hours after cutting the stalk, was followed by the development of functional corpora lutea as evidenced by deciduoma formation. Unfortunately, the experiment was not controlled to show that severing the stalk would, of itself, fail to stimulate the release of the hormone (luteotrophin in this case; it seems probable that LH had already been released since these animals were in full estrus). It should be noted that Friedgood and Bevin (1941) induced pseudopregnancy in the rat by non-specific neck operations.

There is little doubt that estrogen produces certain growth effects on hypophyseal cells independently of nervous influence, since these occur in grafts and in hypophyses whose stalks have been cut (for references see introduction). For the control of many other gonadotrophic phenomena, however, direct hormonal action has been questioned by the investigators who champion the concept of nervous intervention. The operative techniques of these workers have been criticized for damaging the hypophysis. The present work avoids this criticism by replacing surgical procedures with a chemical blocking action which does not alter the structural relationships of the hypophyseal nerves and blood vessels. The resulting complementary evidence of neural involvement in gonadal control of the hypophysis is, therefore, especially significant.

Since removal of the cervical sympathetic ganglia does not impair gonadotrophic function (Vogt, 1931, 1933) the logical pathway for transmission of nervous influence to the hypophysis is the pituitary stalk. Why, then, have so many investigators observed little or no decrement in gonadotrophic function following stalk section? It may be, as Westman, Jacobsohn and Hillarp (1943) suggest, that the

operations were incomplete and that a tuberalis-distalis connection remained intact. However, an alternative explanation is that the final pathway is the portal system and that these vessels may regenerate (Harris, 1948). Regeneration of vessels would also account for the return of function in hypophyseal transplants into the sella (Greep, 1936). Transplants to other sites have not become normally functional (Schweizer, Charipper and Haterius, 1937; Westman and Jacobsohn, 1940). May (1937), however, reported in 2 questionable cases that intraocular hypophyseal transplants in female rats were conducive to normal sex function. The fact that transplant removal did not immediately result in anestrus indicates that hypophysectomy was incomplete. Several workers who claimed that gonadotrophic function was intact after stalk section reported temporary periods of impaired function (Dempsey, 1939; Dempsey and Searles, 1943). Regeneration of portal vessels would have been prevented by Westman and Jacobsohn's (1937) operation in rats; they inserted a metal-foil barrier between the hypothalamus and the hypophysis. Westman, Jacobsohn and Hillarp (1943) emphasized the importance of the tuberalis-distalis connection. If this region was intact the whole infundibular process could be removed without interfering with sex function, but cutting the tuberalis-distalis connection led to gonadal atrophy. Westman, Jacobsohn and Hillarp favored the idea of neural control by tiny nerve fibers which they described entering the distalis through the tuberalis. Their results fit equally well the portal system hypothesis—perhaps even better, for Green (1948) has described as reticulum, fibers in the same location as the Swedish investigators' "nerve" fibers.

Humoral control of the rabbit hypophysis is supported by the results of Markee, Sawyer and Hollinshead (1946–1948). The hypophysis itself was electrically inexcitable to stimuli which, applied directly to the tuberal region of the hypothalamus, produced a maximal discharge of LH. The hormone was also liberated in response to small amounts of adrenaline injected directly into the pars distalis. The rabbit's utilization of an adrenergic agent in the natural stimulus for LH release was evidenced by the ability of Dibenamine to block copulation-induced ovulation (Sawyer, Markee and Hollinshead, 1947). Recently, atropine-block of coitus-stimulated ovulation has given indirect evidence of a cholinergic component in the neurogenic control (Sawyer, Markee and Townsend, 1949). The cholinergic component precedes, and may stimulate, the adrenergic component. Inasmuch as the estrogen-facilitated stimulus in the rat is also blocked by both adrenolytic and anticholinergic agents, the same sequence may hold for the rat. If so, then the influence of estrogen must be exerted no further peripherally than the site of action of the cholinergic component; otherwise atropine would not block it.

In the rat subcutaneous atropine blocks estrogen-induced ovulation and cholesterinization more effectively than does intravenous

Dibenamine. Dibenamine cannot be used subcutaneously because of its extreme toxicity. The reason that intravenous Dibenamine is not completely effective in blocking the stimulus for release of LH when given on the fifth day appears to be that 30 mg./kg. is not above the blocking threshold for all rats. In the fourth day injections, another factor enters in: possible partial inactivation of Dibenamine before the neurogenic stimulation has occurred. This inactivation is indicated by the fact that the same dose on the fifth day is more effective than on the fourth day. The fact that ovulation and preovulatory swelling are blocked more effectively than cholesterol storage confirms Everett's (1947) observation that in the pregnant rat, corpus luteum cholesterinization is a more sensitive indicator of LH release than is ovulation.

In what manner does estrogen operate in influenceng the neurogenic stimulation of LH release? The fact that blocking agents are still effective 20 hours after the estrogen injection, whereas certain vascular effects of estrogen are most pronounced only 6 hours after injection (Astwood, 1939) suggests that the influence of estrogen on the nervous system is a slow indirect type of action. Quite possibly the same sort of influence is exerted in this situation as in the hormonal control of mating behavior. Beach (1948) has suggested that sex hormones influence behavior most likely by altering sensitivity to external stimulation. They are not primary stimulators but they potentiate or diminish neural effects by increasing or decreasing the responsiveness of nervous mechanisms to afferent impulses. Could not estrogen in the rat influence LH release in this manner? This question is discussed at length in the following paper (Everett, Sawyer and Markee, 1949).

If extrinsic factors are primary stimuli for the release of LH in "spontaneously" ovulating species, the only fundamental difference between the rat and the rabbit, in this respect, may be the nature of the receptors and of the afferent neural pathways. It is of interest, in this connection, that estrogen appears to alter the sensitivity of the LH release mechanism in the rabbit as well as the rat. Dury and Bradbury (1943) observed that estrogen lowered the threshold to stimulateon of LH release in the rabbit by copper salts. Klein (1947) reported that copulation in the pregnant or pseudopregnant rabbit, which usually does not induce ovulation, is effective in releasing LH if the rabbit is previously treated with estrogen.

Certain methods of treatment with estrogen in the rat may so alter the sensitivity that the type of receptor mechanism, capable of inducing the release of a hypophyseal hormone, may change. The release of luteotrophic hormone (lactogen) is usually stimulated at copulation, or it may be induced by artificial vaginal stimulation. Estrogen, however, if administered at the proper time, may induce the "spontaneous" release of lactogen as evidenced by pseudopregnancy (see Sawyer and Everett, 1946, for references to the earlier literature).

Progesterone may either inhibit or facilitate release of LH in the rat, depending on dosage and time of injection (Everett, 1948). The effects of this hormone on the hypophysis may also be explained on a basis of altered sensitivity to extrinsic nervous stimulation. Just as estrogen can change the type of effective receptor mechanism so can progesterone. The persistent estrous rat will occasionally ovulate in response to mating or "spontaneously" on injection of progesterone (Everett, 1939). In the rabbit chronic treatment with progesterone inhibits the response (release of LH and ovulation) to mating (Makepeace et al. 1937) and to copper salts (Friedmann, 1941). Unpublished results of Everett and Sawyer indicate that the "stimulating" effects of progesterone in the rat are blocked by adrenolytic and anticholinergic agents.

The concept of a sex center controlling gonadotrophic activity may well be revived. In terms of the more recent evidence it would be a locus in the nervous system at which sex hormones alter the sensitivity to extrinsic stimuli and thus influence hypophyseal secretion. The "sex center" for control of mating behavoir in the cat was localized near the rostral end of the mesencephalon (Bard, 1940). In view of gonadotrophic disturbances resulting from hypothalamic lesions (Dey, 1943) and the extreme sensitivity of hypothalamic nuclei in experiments on the electrical stimulation of the release of LH (Haterius and Derbyshire, 1937; Harris, 1937; Markee, Sawyer and Hollinshead, 1946; Harris, 1948), the hypothalamus would appear to be the most likely site of the gonadotrophic sex center.

SUMMARY AND CONCLUSIONS

It has been generally accepted that, in polyestrous animals, estrogen stimulates the release of luteinizing hormone by a direct action on adenohypophyseal cells. The present study, employing 60 mature female rats, introduces convincing evidence that the nervous system is involved in the estrogen-induced discharge of hypophyseal LH in the rat.

In the pregnant rat, injection of estrogen on day 4 is followed within 48 hours by ovulation and storage of cholesterol in the corpora lutea of pregnancy. Both of these phenomena are indicative of LH release, and they may be duplicated by injecting LH on the fifth day of pregnancy. If the potent adrenergic-blocking agent, Dibenamine, is injected prior to, or as much as 20 hours after estrogen administration, ovulation and luteal cholesterinization are prevented in a high proportion of the cases. The effects of injected LH are, however, not counteracted by Dibenamine. The anti-cholinergic drug atropine also allows injected LH to manifest its effects but, like Dibenamine, blocks estrogen-induced ovulation and luteal cholesterinization.

It has been amply demonstrated by experiments in the rabbit that Dibenamine and atropine prevent the release of luteinizing hormone only by blocking neurogenic stimuli from reaching the hypophysis.

The drugs interfere neither with the mechanism of glandular discharge of the hormone nor with the action of LH on its target organs. The present experiments demonstrate that estrogen-induced release of LH in the rat is blocked by the anti-adrenergic and anti-cholinergic drugs. It is therefore concluded that this effect of estrogen on the rat hypophysis is mediated, at least in part, by the nervous system. It is suggested that estrogen exerts its action by facilitating, or lowering the threshold to, extrinsic stimulation of a gonadotrophic sex center probably located in the hypothalamus.

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A NEUROGENIC TIMING FACTOR IN CONTROL OF THE OVULATORY DISCHARGE OF LUTEINIZING HORMONE IN THE CYCLIC RAT[1]

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INTRODUCTION

IT WAS reported in the preceding paper (Sawyer, Everett and Markee, 1949) that a neural factor is involved in the release of LH[2] from the female rat hypophysis in response to estrogen treatment, thus extending to a "spontaneously" ovulating species principles which had been previously disclosed in the rabbit (Markee, et al., 1946, 1948; Sawyer, et al., 1947, 1948). Although estradiol benzoate injected on the fourth day of pregnancy in the rat regularly induces ovulation within 48 hours (Everett, 1947), this effect could be prevented in most cases by Dibenamine or atropine, administered as late as 20 hours after the estrogen. It was proposed that estrogen modifies the threshold of activation of an hypothalamic center, or of certain afferent pathways leading to the "center," and that a chief difference between the rabbit and rat lies in the particular afferent pathways concerned.

While in the domestic rabbit the most important nervous pathways concerned with the LH-release mechanism are those which are activated directly or indirectly by copulation, these are generally thought to have no influence on discharge of luteinizing hormone in the rat. Here, the only exteroceptive pathways known to be concerned in regulation of the onset of estrus and of ovulation time are optic (Hemmingsen and Krarup, 1937; Browman, 1937) and perhaps thermal (Lee, 1926; Browman, 1943, b; Dempsey and Searles, 1943). In the absence of light the normal nocturnal activity and estrous rhythms can be maintained or reversed by maintaining or reversing the 24-hour rhythm of temperature fluctuation (Browman, loc. cit.). Conceivably, as estrogen secretion increases, the thresholds in these

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[2] The expression LH is used in this paper in the conventional sense (cf. Hisaw, 1947), recognizing that FSH also enters into preovulatory stimulation of follicles. The effects with which we are concerned here may largely express quantitative rather than qualitative differences in gonadotrophin secretion.

systems and in a variety of others are lowered (Beach, 1948), eventually resulting in a discharge from the "sex center" in the hypothalamus.

It was obviously important to extend the experiments to the normal cyclic ovulation. The results of that study are the subject matter of the present report. It will be demonstrated that cyclic ovulation can be blocked by injection of either Dibenamine or atropine as late as 2 P.M. on the day of proestrus. Dibenamine gives somewhat less constant results than atropine. Injections of either drug at 4 P.M. are almost entirely ineffective, indicating that in our colony neurohumoral stimulation of the anterior lobe occurs sometime between 2 and 4 P.M., about 9 to 11 hours before ovulation. A preliminary account of a part of these results was recently published (Everett, Sawyer and Markee, 1948).

MATERIALS AND METHODS

The techniques of administration of Dibenamine and of atropine were those described by Sawyer, Everett and Markee (1949). Dibenamine was injected intravenously, the concentration being 10 mg./ml. and the standard dose 30 mg./kg. Atropine sulphate at a concentration of 70 mg./ml. in Ringer-Locke solution was injected subcutaneously, the standard dose being 700 mg./kg.

In this study 87 cyclic female rats of the inbred Vanderbilt (Osborne-Mendel) strain were used. The definitive experimental groups, comprising 68 rats, include only animals having histories of regular 4-day cycles (substrain Va). The remaining 19, used during the exploratory stage of the work, were 5-day cyclic females mostly of the substrain Vc. These two substrains have recently been described (Everett, 1948) with respect to predictability of length of cycle and of the time of ovulation. It was stated that in the Va substrain, in which most cycles are 4 days long, if a given animal has experienced 2 observed 4-day cycles in sequence the probability is about 93% that the next cycle will also be of that length. In the Vc substrain, on the other hand, most females are 5-day cyclic. After 2 successive observed cycles of that duration the probabilities are about 90% that the next cycle will be similar, 94% that it will not be prolonged. These probabilities are further improved by selection of animals showing characteristic proestrous vaginal smears on the morning of expected proestrus (8–10 A.M.). In the 4-day cyclic group 25 to 30% of the animals still present a few leucocytes in the smears at this time. Where such rats were used the degree of vaginal intumescence was employed as an additional criterion and an early afternoon smear was prepared in confirmation. No attempt was made to use the criterion of psychic estrus, as the systemic effects of the drugs are such that no reliance could be placed upon it. The time of ovulation in the 4-day Va rats is between 1 and 2:30 A.M. during the early morning of the day following proestrus. Comparable data on 5-day cyclic rats have not been obtained, but the histological appearance of their new corpora lutea during the late morning after ovulation (vaginal stage III) strongly indicates that here also ovulation occurs not long after midnight.

The regularity of cycles and the constantly close relationship of ovulation

time to hours of the day may be ascribed to the genetic homogeneity of the animals and to the uniformity of the diurnal rhythm of lighting. The colony lighting is entirely controlled by a time switch, the lights being on for 14 hours each day. The period of darkness is thus 10 hours.

The experimental procedure in general outline was as follows: One or the other drug was administered at a certain hour of the day of proestrus (8–9 A.M., 2 P.M., 4 P.M., 5 P.M., or 6:45 P.M.). Autopsy was routinely performed on the following morning between 8 and 10:30 A.M., after killing the animal with illuminating gas. (In a few cases, as specified later, the experiments were continued longer.) The status of the uteri was noted with respect to hyperemia, reactivity to manipulation and, especially, degree of distention and fluid content. Both ovaries with tubes attached were removed to a dish of physiological saline. Under a dissecting microscope the presence or absence of dilatation of the ampullae of the oviducts was observed. The ampullae were excised and transferred to a drop of saline on a microscope slide. After straightening their coils, a cover slip was applied and the preparation was observed under the low power of a compound microscope by transmitted light. Tubal ova, if present, are usually readily visible by this means. Each ovary was then carefully freed from surrounding structures by incising the capsule and clipping the mesovarium with iridectomy scissors. The status of follicles was observed—whether clear or hyperemic, degree of hyperemia, approximate diameter, etc. Rupture points were looked for. The appearance of the interstitial tissue was recorded—whether markedly fatty as it is normally during proestrus, or dull and watery, indicative of the cholesterol depletion which takes place during estrus (see below). The corpora lutea of the preceding cycle were observed for the apparent degree of fat content. On the day after ovulation they are normally distinctly creamy, in contrast with a relatively pallid aspect during proestrus (Everett, 1945).

Routinely, one or both ovaries were preserved in Zenker's fluid for paraffin imbedding and serial sectioning. Where a full set of tubal ova was found (6 to 11), such preparations often seemed unnecessary and in only a few such instances did we serially section even one ovary. In representative cases in most of the experimental groups portions of one or both ovaries, or an entire ovary, were fixed in 10% formalin. Frozen sections were cut at 30μ thickness and were examined by the Schultz test for cholesterol content of the interstitial tissue and corpora lutea (Everett, 1945, 1947). One index of LH action on the ovary is depletion of interstitial cholesterol content (Claesson and Hillarp, 1947), tending to be most pronounced near ripe follicles and in the vicinity of new corpora lutea (Everett, 1947 and unpublished). Increased cholesterol storage in corpora lutea of the previous cycle is also a measure of LH action under certain conditions (Everett, 1945, 1947). Thus, our 3 criteria of LH secretion are: (1) ovulation or preovulatory swelling; (2) cholesterol depletion in the interstitium, as evidenced grossly by a dull, watery appearance or microscopically by a negative or very weak Schultz test; (3) cholesterol storage in the corpora lutea of the preceding cycle, as evidenced by a strongly positive Schultz reaction or simply a "creamy" appearance at the time of autopsy.

RESULTS

Dibenamine

Since in pregnant rats treated with estrogen, Dibenamine had been successful in preventing ovulation when injected at 8 A.M. on the morning before expected ovulation (Sawyer, Everett and Markee, 1949), this hour on the day of proestrus was chosen for the preliminary trials in cyclic rats. Of the 10 animals in this initial experiment 6 were 5-day cyclic Vc rats and 4 were 4-day cyclic Va rats, all having proestrous smears at injection time (8 to 9:30 A.M.). At autopsy on the following morning none had tubal ova. No rupture points were visible under the dissecting microscope. With the exception of 2 of the 4-day rats, histological study of the ovaries revealed no evidence of preovulatory swelling of the follicles. In the 2 exceptional cases a few follicles were found to be in early stages of swelling with first polar body divisions in progress or complete. Since the control experiments with LH in the pregnant rat (Sawyer, Everett and Markee, loc. cit.) had shown that Dibenamine does not interfere with the action of LH on the ovary, the present results left no doubt that adrenergic stimulation of the hypophysis occurs during the day of proestrus and that this stimulation is essential for secretion of LH sufficient to induce ovulation. By treatment at various hours of the day it should thus be possible to determine time limits within which the stimulation occurs.

Because of the greater availability of Va stock and the more complete information about ovulation time in the 4-day cycle we subsequently confined our attention principally to 4-day cyclic animals. The 8–9 A.M. injection group was increased by 12 rats (9 autopsied at the usual time and 3 autopsied about 48 hours after injection). Thus a total of 16 4-day cyclic rats was treated between these hours. In table 1 are listed the 13 cases which were autopsied 24 hours later. Ovulation was blocked in 9 cases, but the presence of preovulatory swelling in a few follicles in 2 instances (mentioned in the preceding paragraph) indicated a slight amount of LH secretion. Two rats had 2 and 3 tubal ova, respectively. In one of these rats, sections of both ovaries contained 2 rupture points, 2 follicles in early swelling and 5 unstimulated follicles with resting ovocyte nuclei. In the other in which the ova were confined to the right tube, sections of the left ovary contained 3 "unstimulated" follicles and none which had advanced beyond that stage. These 2 cases, therefore, also indicate less than the normal LH secretion. The other 2 rats were judged to have fully ovulated, although one had only 5 tubal ova, 4 on the right and 1 on the left. Her left ovary contained only 1 ruptured follicle, but of 4 follicles with resting ovocyte nuclei, 2 exhibited localized theca luteinization. It is possible that this case also represents diminished LH secretion resulting from the treatment, but the uncertainty led

to its classification as complete ovulation. The other rat had 8 tubal ova and her reproductive tract otherwise gave a completely normal picture of late estrus.

Of the 3 rats which were autopsied 48 hours after the 8 A.M. injection, 2 failed to ovulate and 1 had a set of new corpora lutea. One

TABLE 1. SUMMARY OF DATA SHOWING STATUS OF FOLLICLES AND EXTENT OF OVULATION ON THE MORNING AFTER TREATMENT WITH DIBENAMINE OR ATROPINE

DIBENAMINE

Hour injected	No. of rats	Tubal ova			Follicles			Ovulation	
		None	Few	Many	No swelling	Early swelling	Late swelling	In progress	Complete
8–9 A.M.	13	9	3	1	7	2		2	1 1
2 P.M.	19	12	4	[3][1]	7	2	2	1 4	[3][1]
4 P.M.	10	3	1	6		1	1	1	1 6
5 P.M.	4	1[2]		3			1[2]		3
6:45 P.M.	4			4					4

ATROPINE

Hour injected	No. of rats	Tubal ova			Follicles			Ovulation	
		None	Few	Many	No swelling	Early swelling	Late swelling	In progress	Complete
8 A.M.	5	5			5				
2 P.M.	5	5			5				
4 P.M.	5		1	4					1 4

[1] Autopsied at 4 P.M., ca. 26 hours after injection.
[2] Found dead but still warm at 7·45 A.M.

ovary of each rat was serially sectioned, disclosing in the anovulatory cases "unstimulated" follicles, nearly all of which were atretic. The new corpora in the third animal were somewhat less advanced than normal for this stage of the cycle. Ovulation had probably been retarded. The vaginal smears in all 3 rats were metestrous, a marked invasion of leucocytes being seen in all. Thus, the interruption of estrogen secretion is indicated, a matter to be discussed later.

As shown in table 1, 19 4-day cyclic rats were injected at about 2

P.M. on the day of proestrus. Nine of them were autopsied between 8 and 9 A.M. the next day, 1 at 12:45 P.M. and 9 at about 4 P.M. Tubal ova were absent in 5 of the first 9, in the 12:45 case and in 6 of the 9 killed in late afternoon. Seven of these 12 animals gave no indication of preovulatory swelling. Three (among the morning autopsy series) exhibited preovulatory swelling in variable degree. The rat autopsied at 12:45 had 3 very recently ruptured follicles which would undoubtedly have been in late swelling if she been killed as early as the others; she is so classified in table 1. One rat in the morning group had 5 follicles in late swelling and a single ruptured follicle, but the ovum had apparently not yet reached the tube. Four others in the morning group had 1 or 2 tubal ova and histological study of their ovaries disclosed from 1 to 4 ruptured follicles and a variety of stages of preovulatory swelling. Three of the rats killed at 4 P.M. had completely ovulated (10 to 11 ova). Although they are classified in table 1 as complete ovulations, they would not necessarily have been so had they been autopsied at the morning hour. In one of them tertiary follicular fluid had not yet been formed in large amount, strongly suggesting delayed ovulation.

The group injected at 4 P.M. comprises 10 rats (table 1), all of which were autopsied the following day between 8 and 9 A.M. In none of these was LH release completely blocked, although tubal ova were absent in 3 animals. All 3 had follicles in preovulatory swelling. In one of them, 2 follicles had already ruptured and in another all 10 follicles were very near the time of rupture. A fourth rat had 5 tubal ova and 7 rupture points. The remaining 6 rats had from 7 to 10 tubal ova and full sets of new corpora lutea which were histologically identical with those normally found at this time in 4-day cyclic rats.

The results of injection at 5 and 6:45 P.M. may be considered together. Four rats were injected at each of these hours. One animal in the 5 o'clock group was found dead but still warm shortly before 8 A.M. the next morning. She was autopsied immediately. No tubal ova were found, nor ruptured follicles, but histological study of one of the ovaries disclosed 5 follicles in late preovulatory swelling. The vaginal smear was still in late proestrus. It is possible that the retardation of follicle maturation and of the vaginal changes was due to non-specific systemic factors associated with the moribund condition during the night. The other 7 rats had fully ovulated, having from 6 to 10 tubal ova.

In the experiments with pregnant rats treated with estrogen (Sawyer, Everett and Markee, 1949), the occurrence of subcutaneous "mucoid" as a sequel to Dibenamine treatment was closely correlated with the blocking effect of this agent. In the present experiments, however, such correlation was less complete. In 4-day cyclic rats of the 8–9 A.M. and 2 P.M. injection groups, among 26 cases in which our records include reference to mucoid and in which ovulation was either

completely or partially blocked, 17 rats had mucoid at autopsy while 9 had none. Its presence or absence does not, therefore, appear to be an acceptable criterion of adequacy of Dibenamine dosage under these conditions.

Atropine

It was apparent from the results with Dibenamine that injections at 2 P.M. or earlier usually result in either complete or partial block of LH release, while injections at 4 P.M. or later usually do not interfere with the release mechanism at least so far as ovulation is concerned. Evidently adrenergic stimulation of the hypophysis occurs mainly in the interval between 2 and 4 P.M. in 4-day cyclic rats in this colony. Since a cholinergic link in neurogenic stimulation of LH release has been indicated in the rabbit (Sawyer, Markee and Townsend, 1949) it was of interest to determine whether a cholinergic link also occurs in the cyclic rat. Concurrent experiments with pregnant rats (Sawyer, Everett and Markee, 1949) demonstrated that ovulation after estrogen administration is prevented by atropine injected on the day before ovulation is expected. Yet atropine, like Dibenamine, does not prevent ovulation in response to injected LH.

Five 4-day cyclic rats were injected with atropine at 8 A.M. On the following morning the vaginal smears were still proestrous. At autopsy about 24 hours after injection the reproductive tracts were uniformly characteristic of a proestrous rat. No tubal ova were to be seen (table 1). The follicles were large, but not hyperemic. In histological section no evidence of normal preovulatory swelling was seen. In one rat a single follicle among 9 showed the first maturation division in progress, but as mitotic divisions could not be found in the granulosa this follicle was judged to be atretic. In a second rat a single follicle among 9 was atypical. The status of the ovocyte nucleus could not be determined because of a few missing sections, but there was no secretion of secondary liquor about the cumulus. The granulosa was compact and of irregular thickness. Its cells were disoriented as in late preovulatory swelling and mitotic figures were most unusually numerous. The basement membrane was intact. Whether this follicle represents slight stimulation we cannot state. All others (7–8 in each of the 5 rats) appeared to be normal, growing follicles with resting ovocyte nuclei.

It was apparent from these results that a cholinergic link exists in the LH-release mechanism of the cyclic rat. To estimate the time limits within which this part of the mechanism operates, atropine was injected into five 4-day cyclic rats at 2 P.M. on the day of proestrus and into 5 others at 4 P.M. (table 1). The animals were killed and autopsied the following day between 8 and 9:15 A.M. In the 2 o'clock group not one rat had tubal ova. Their ovaries presented the characteristic aspect of ovaries seen in normal proestrus, with large clear follicles. Histological study showed these to be healthy, growing follicles with

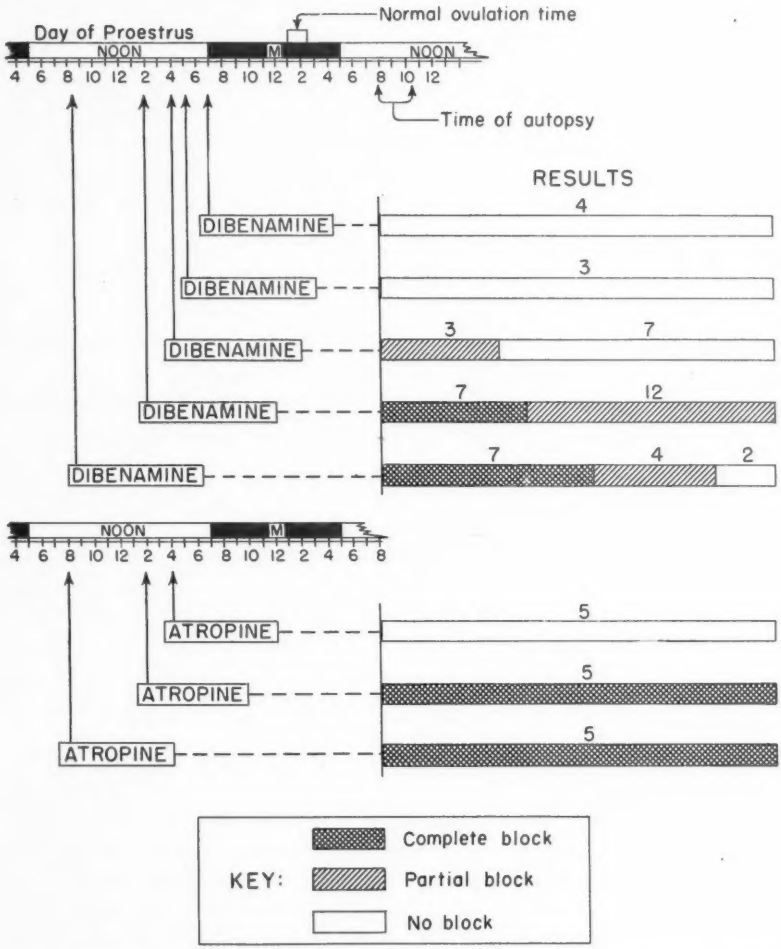

FIG. 1. Graphic representation of the degrees of blocking of the LH-release mechanism obtained by injecting Dibenamine or atropine at various hours of the day of proestrus, in 4-day cyclic rats. The numbers over the various segments of the bars under "RESULTS" indicate the respective numbers of rats represented by the proportional lengths of the segments.

resting ovocyte nuclei in all instances. In the 4 o'clock injection group the results were quite different (table 1). Four of the 5 rats had full sets of tubal ova (7–10). The fifth rat had 3 observed tubal ova, 1 on the right and 2 on the left. Her right ampulla, however, was exceedingly dilated and the right ovary when examined histologically was found to contain 5 new corpora lutea in a comparable stage of development with those in the other 4 rats.

In figure 1 the above data from the Dibenamine and atropine se-

ries are summarized in graphic form, with respect to the apparent degree of block of the LH-release mechanism. With Dibenamine, partial or complete block is the rule when injections are made at 2 P.M., or earlier, on the day of proestrus. With atropine, complete block is the rule under like conditions. With Dibenamine, ovulation usually proceeds unhampered following injections at 4 P.M. or later. The same is true with atropine. No real difference can be claimed between the 4 P.M. Dibenamine series and the 4 P.M. atropine series, on the basis of the present numbers of cases.

TABLE 2. CORRELATION BETWEEN VAGINAL SMEAR RETARDATION AND EXTENT OF BLOCKING BY DIBENAMINE

Vaginal smear stage (morning of autopsy	Hours of Dibenamine injection (preceding day)	Degree of blocking, measured by ovulation		
		Blocked (no. of rats)	Partially blocked (no. of rats)	No block (no. of rats)
Late proestrus	8–9 A.M.	1	3	1(?)[1]
	2 P.M.	5	7	
	4 P.M.		4	
	5 P.M.		1[2]	
	All hours	21		1
Estrus	8–9 A.M.	8	2	1
	2 P.M.	2	2	
	4 P.M.			6
	5 P.M.			3
	6:45 P.M.			4
	All hours	14		14

[1] 5 Tubal ova, 2 follicles partly luteinized but not ruptured.
[2] Found dead but still warm at 7:45 A.M.

Correlation between status of ovaries and status of vagina and uterus. In the atropine series there was a clear-cut correlation between incidence of full ovulation and of cornified vaginal smears. All rats injected with atropine at 2 P.M. or earlier had proestrous smears the next morning. The one animal in the 4 P.M. injection group which had a reduced number of tubal ova at autopsy time, also had a late proestrous smear. The others were fully cornified.

In the Dibenamine series, the correlation was somewhat similar but less clear-cut (table 2).[3] The principal lack of conformity was in the 8–9 A.M. injection group where 8 of 9 anovulatory rats,[4] showing no preovulatory swelling, were nevertheless fully cornified 24 hours after injection. Considering all Dibenamine groups together, the incidence of proestrous smears was greatest in the animals showing partial block, strangely enough. Among the "blocked" and "partially

[3] The cases admitted to tables 2 and 3 are not in all instances those recorded in table 1. For various reasons a few of the latter are excluded and certain others are pertinent.
[4] Including 2 rats autopsied at 48 hours.

blocked" groups combined, 21 of 35 rats had proestrous smears on the morning after injection. On the other hand, 14 of 15 rats in which ovulation was not significantly delayed were fully cornified.

As regards the uteri in the Dibenamine series, a similar correlation was found (table 3)[3] between uterine distention and the prevention or retardation of follicle maturation. Thus, among 24 "blocked" or "partially blocked" rats, 19 exhibited some degree of uterine distention, while this was seen in only 4 out of 15 of the "not blocked" group. In

TABLE 3. CORRELATION BETWEEN UTERINE DISTENTION AT AUTOPSY ON THE MORNING AFTER DIBENAMINE INJECTION, AND EXTENT OF BLOCKING

Status of uteri	Hour of Dibenamine injection (preceding day)	Degree of blocking, measured by ovulation		
		Blocked (no. of rats)	Partially blocked (no. of rats)	No block (no. of rats)
Distended (proestrous)	8–9 A.M.	5	3	1(?)[1]
	2 P.M.	1	8	
	4 P.M.		2	2
	6:45 P.M.			1
	All	6	13	4
Not distended (estrous)	8–9 A.M.	1	1	1
	2 P.M.		1	
	4 P.M.		2	4
	5 P.M.			3
	6:45 P.M.			3
	All	1	4	11

[1] 5 Tubal ova, 2 follicles partly luteinized but not ruptured.

the atropine series all of the 8 o'clock injection group and 4 of the 2 o'clock group had distended uteri. In the 4 o'clock group 2 rats had slightly distended uteri, including the animal in which the vaginal smear was proestrous. In the other 3 no distention was seen.

Status of interstitial tissue and corpora lutea of the preceding cycle. There was a close correlation between the degree of follicle maturation or ovulation and the degree of depletion of interstitial lipid. In the Dibenamine group, all "blocked" rats had a fatty interstitium. Fifteen of 20 "partially blocked" rats were similar, while 5 showed at least local depletion near ruptured and preovulatory follicles. Among 15 rats which were "not blocked," interstitial lipid was partially or strongly depleted in 14 cases. The atropine series gave similar results.

Cholesterol storage in the corpora lutea of the preceding cycle was surprisingly rare, even in the animals injected at 4 P.M. or later, in both the Dibenamine and the atropine series. Schultz-positive corpora lutea were found in only 3 Dibenamine-treated rats in the definitive (4-day cyclic, 24-hour autopsy) series: 1 of the completely ovulated rats of the 8–9 A.M. injection group, 2 completely ovulated rats of the

4 P.M. group. The corpora were uniformly Schultz-negative in the 7 rats which ovulated in spite of injection of Dibenamine at 5 to 7 P.M. Where autopsy time was delayed until late afternoon, 26 hours after 8 A.M. Dibenamine injection, or longer (48 hours), 3 of the 4 rats which ovulated had distinctly fatty corpora lutea. Schultz tests were not performed. In the atropine series the corpora were grossly pallid in all cases. Schultz tests were performed on corpora from 3 of the 4 o'clock injection group and from one each of the other groups. All were negative.

DISCUSSION

Before considering the broad implications of the above experiments certain details require evaluation. While atropine proved to be completely effective in blocking ovulation when injected at 8 A.M. or 2 P.M., Dibenamine completely blocked only 14 of the 32 4-day cyclic rats at these hours. These facts might be construed to mean that in the rat the adrenergic component of the LH-release mechanism precedes the cholinergic. This interpretation is contradicted, however, by the 4 P.M. data and by unpublished data based on the phenomenon of advancement of ovulation time by progesterone. When progesterone is administered on the third day of diestrus in 5-day cycles, ovulation time is advanced about 24 hours (Everett, 1944, a; 1948). Injection of Dibenamine just after progesterone, or 4 hours before, completely blocked this effect in a number of cases, but in several instances a few follicles were found in preovulatory swelling (on the morning of normally expected proestrus).

The present evidence indicates, then, that Dibenamine in the cyclic rat, as in the pregnant, estrogen-treated rat (Sawyer, Everett and Markee, 1949), is not a perfect blocking agent. The partial effects observed in cyclic rats were, however, expressed somewhat differently. Where in the pregnant rats subovulatory LH activity was detected by cholesterinization of the pregnancy corpora lutea, in cyclic rats diminished LH secretion was made evident chiefly by retardation of follicle maturation. The degree of lipid depletion in the interstitial tissue proved, in the cyclic rat, to be a better indicator of LH than corpus luteum cholesterinization. The general absence of luteal cholesterol storage on the morning after Dibenamine or atropine treatment, even when full ovulation occurred, may, of course, indicate moderate lowering of LH secretion. It is significant that in 3 of the 4 ovulated rats, in which autopsy was delayed considerably beyond the usual time after Dibenamine treatment, the corpora lutea of the previous cycle were fatty. It thus seems probable that many of the negative cases in the definitive series would have become Schultz-positive a few hours later. More information is needed about the respective time-response relationships of ovulation, interstitial cholesterol depletion and luteal cholesterinization to various quantities of LH.

Considering the 8–9 A.M. injection group as a measure of the in-

complete blocking effects of Dibenamine and thus a control for the 2 P.M. group, the Dibenamine and atropine series are in close agreement. They demonstrate that in the particular strain of rats with which we worked, during proestrus of the 4-day cycle, a neurogenic stimulus passes to the hypophysis between 2 and 4 P.M. in most animals. This stimulus has both cholinergic and adrenergic components. The evidence from the rabbit (Sawyer, Markee and Townsend, 1949) would indicate that the cholinergic component precedes the adrenergic. We can only assume that the same relationship is true in the rat. We must also draw on the rabbit studies for the most crucial evidence that neither Dibenamine nor atropine impairs the ability of the hypophysis to liberate LH. If injection of either agent in rabbits is delayed for even a minute after coitus, ovulation proceeds without interference. Hence LH liberation *per se* is not affected, for that process is known to require about an hour in the rabbit (Fee and Parkes, 1929).

The fact that the majority of rats in which ovulation was delayed or completely prevented also experienced retardation of the vaginal smear sequence is of great importance in itself. This inhibition of vaginal smear changes in conjunction with blocked ovulation constitutes the reverse of conditions described in 5-day cyclic rats in which ovulation time was advanced 24 hours (Everett, 1944, a; 1948) by progesterone therapy. Under those conditions the vaginal sequence was accelerated; here it is retarded. The significance of these effects has been discussed in the recent report of the progesterone experiments (1948).

The demonstration of a chronologically limited neurogenic stimulus for the ovulatory discharge of LH from the adenohypophysis, for the first time indicates a means by which at least one of the chronologic features of the polyestrous cycle may be synchronized with environmental rhythms. Synchronization of the cyclic activity of the hypophysis-gonad system with environmental factors is a well recognized fact in many species of mammals and lower vertebrates (Marshall, 1936, 1942; Bissonnette, 1936; Beach, 1948). Such effects are best seen in the seasonal breeders, in several of which it has been well established that conditions of illumination are of paramount importance. Among constant breeders the effects of environment are generally more obscure. Among polyestrous, spontaneously ovulating species it has usually been thought that the rhythmic interplay between hypophysis and ovary proceeds as a humoral mechanism largely without neural intervention.

Nevertheless, there are clear indications that the cycles of rats, mice, guinea pigs and hamsters are synchronized with diurnal rhythms of illumination and temperature. Thus it has been well recognized that rats usually ovulate and exhibit maximal estrus during the dark period. As a corollary of this fact, Hemmingsen and Krarup state: (1937, p. 28) "The length of an oestrous cycle must always be a whole number of days." These authors were able to reverse the nocturnal

rhythms of running activity and of maximal estrus by keeping rats in darkness during the solar day and illuminating them at night. Similar effects have been described in the mouse (Snell, et al., 1940; Gresson, 1940). Snell, et al. reported, furthermore, that in mice under such reversed lighting, ovulation usually occurs between the artificial "midnight" and 3 "A.M." It has been stated that ovulation in the golden hamster occurs quite precisely between midnight and 2 A.M. (Ward, 1946). The guinea pig has been reported to exhibit maximal estrous behavior during the night (Young, Myers and Dempsey, 1933).

In our inbred strain of rats under regulated conditions of illumination, animals which are carefully selected for regularity of cycles ovulate within the predictable time limits of 1:00 to 2:30 A.M. The neurogenic stimulus to the hypophysis reaches the gland, as we have now shown, between 2 P.M. and 4 P.M. of the preceding afternoon, roughly 10 to 12 hours before ovulation. This interval is remarkably similar to that between copulation and ovulation in the rabbit (Fee and Parkes, 1929; Waterman, 1943, and refs. cited there). In the rat the actual hours reported here for both stimulation and ovulation may well vary in other strains and under other conditions. In colonies in which lighting is not controlled a considerable irregularity may occur and where periods of controlled lighting per day are other than 14 hours deviations may be expected from the time limits which we observed.

In the preceding article of this series (Sawyer, Everett and Markee, 1949) it was proposed that the concept of a "sexual center" in the hypothalamus be revived. The present evidence gives additional support for this view and allows us to speculate upon some of the functional characteristics of such a center in the rat, under different hormonal and environmental conditions.

A principal factor leading to discharge of the "ovulation stimulus" from the "center" is probably an elevated estrogen level *per se* or some metabolic condition associated with elevated estrogen. This may act directly in the hypothalamus or may modify the afferent impulses feeding into it (Beach, 1948). Progesterone may synergize importantly with estrogen in this action, for under certain conditions progesterone facilitates ovulation in this species (Everett, 1948). It is noteworthy that Kent, (1948) reports that castrated, estrogen-primed hamsters are brought into psychic estrus as early as 20 minutes after introduction of a minute amount of progesterone into the cerebral ventricles.

The rhythm of daily illumination is concerned in the rhythmicity of the "center." This effect does not appear to be a direct one, however, for when rats are placed in nearly constant darkness they remain regularly cyclic (Browman, 1937–1943), even when temperatures are

MEASUREMENTS IN ABNORMAL PELVES

FOR A PELVIS TO BE CONTRACTED THE BISISCHIAL MUST BE EIGHT CENTIMETERS OR LESS. THE DIAGONAL CONJUGATE MUST BE ELEVEN CENTIMETERS OR LESS IN A SIMPLE FLAT PELVIS OR ELEVEN AND FIVE-TENTHS CENTIMETERS IN A GENERALLY CONTRACTED PELVIS. ALL OTHER MEASUREMENTS MERELY DETERMINE THE TYPE OF PELVIS. IF THE POSTERIOR SAGITTAL PLUS THE BISISCHIAL DIAMETER TOTALS FIFTEEN CENTIMETERS OR LESS, THE PELVIS IS AS A RULE CONTRACTED.

DIAMETERS	NORMAL	RACHITIC FLAT	RACHITIC GENERALLY CONTRACTED	SIMPLE FLAT	GENERALLY CONTRACTED	FUNNEL	GENERALLY CONTRACTED FUNNEL
PERCENTAGES	87%	1%		2%	4%	5%-6%	1%
INTERSPINOUS	26.00 CM.	N OR >	<	N OR >	<	N	<
INTERCRISTAL	28.00 CM.	N	<	N	<	N	<
EXTERNAL CONJUGATE	21.00 CM.	<	<	< BUT NOT LESS THAN 18 CM. AS A RULE	<	N	<
DIAGONAL CONJUGATE	12.75 CM.	11 CM. OR LESS	11 CM. OR LESS	11 CM. OR LESS	11.5 CM OR LESS	N	11.5 CM. OR LESS
BISISCHIAL	10.00 CM.	>	>	N	<	8 CM. OR LESS	8 CM. OR LESS
ANTERIOR SAGITTAL	5.50 CM.	N	<	N	<	N	<
POSTERIOR SAGITTAL	7.8 CM.	N	<	N	<	>	N
ANTERO-POSTERIOR	11.50 CM.	N	< OR N	N	<	>	N
SACRAL CURVATURES	NORMAL	VERTICAL CONCAVITY > LATERAL CONCAVITY < OR CONVEX		N	N	N	N
TRANSVERSE OF INLET	13.50 CM.	> OR N	N OR <	> OR N	N OR <	N	N OR <
PELVIC INCLINATION	60°	> DUE TO LORDOSIS					
SACRUM	NORMAL	BROAD LESS CONCAVE SHORTER			N = NORMAL	HIGH ASSIMILATION OFTEN 6 SACRAL VERTEBRAE	

constant. In fact, they often retain preceding nocturnal rhythms of activity and estrous behavior. Such effects of illumination are mediated by the optic nerve (Browman, loc. cit.). Subservient to rhythms of light and darkness, diurnal rhythms of temperature are also important in conditioning the activity and estrous rhythms (Browman, 1943, b). This becomes apparent during constant darkness or after enucleation or cutting the optic nerve.

Certain effects of lighting may be quite immediate. Exposure of rats to continuous light often induces the persistence of follicles and of vaginal estrus (Browman, 1937–1943; Hemmingsen and Krarup, 1937). In animals which are especially susceptible these effects may begin during the estrus which immediately follows the introduction of continuous light (Everett, 1942 and unpublished). Such responses to continuous light also depend on intact optic nerves (Browman, loc. cit.) and probably to a large extent upon the hormonal status. Progesterone facilitates the cyclic action of the LH-release mechanism during continuous light (Everett, 1940, a). Indications are that in "light-susceptible" rats the hypothalamic mechanism for the stimulation of LH-release is refractory to estrogen, perhaps because of faulty progesterone secretion (Everett, 1942–1948).

Other effects of lighting are delayed in their expression. Reversal of estrus by reversed day-night requires about 10 days (Hemmingsen and Krarup, 1937). A certain strain of rats (DA) in our colony tends to become anestrous after the daily light ration is reduced to less than 10 hours. This effect is usually first indicated, by slightly prolonged diestrous intervals, about 15 days after the new conditions are established. The full effect may not be registered until 30 days or later (Everett, 1942 and unpublished). Another curious effect of short day-length in this strain is observed in older females which have become "spontaneously" persistent-estrous during 14-hour days. Two weeks to a month after the establishment of "short days," cycles often reappear. These cycles are accompanied by renewed corpus-luteum formation (unpublished).

In the phenomenon of persistent estrus, either "spontaneous" or light-induced, we are dealing with a condition which is not uncommon in older females of many species of mammals (follicle retention cysts with chronic estrogen secretion). It would seem that one direct cause of such aberrant occurrences is the failure of the hypothalamus to discharge an adequate stimulus to the hypophysis. As a result the hypophysis continues more or less steadily to secrete only small amounts of gonadotrophin which are nevertheless sufficient to maintain estrogen secretion. This steady state is normal in such species as the rabbit, but subject there to interruption by the imposition of a set of afferent neural impulses associated with copulation. Significantly the steady state of persistent estrus in the rat may similarly be

interrupted by imposition of new conditions: (1) occasionally by copulation (Everett, 1939 and unpublished); (2) by shortened day-length as mentioned above; (3) by progesterone therapy (Everett, 1940, b; 1943); and (4) by daily treatment with desoxycorticosterone or with small doses of testosterone propionate (Marvin, 1947, 1948).

Whether secretion of gonadotrophin during such steady states requires neurogenic stimulation remains uncertain. It has been reported that moderate secretion can occur in hypophyses transplanted to the kidney or anterior chamber of the eye (Martins, 1936; Schweitzer, Charipper and Haterius, 1937). However, Westman and Jacobsohn hold to the view that the central nervous system maintains a steady trophic action on the hypophysis which is essential for adequate synthesis of gonadotrophin (see references and discussion in Sawyer, Everett and Markee, 1949). With regard to this question it is perhaps significant that in 3 of the present experiments, in which autopsy was delayed until 48 hours after 8 A M. Dibenamine injection, the vaginal smears became leucocytic not only in the rat which ovulated but in the other two as well. In the latter, marked follicular atresia was found. It would appear that in these two instances Dibenamine not only prevented the ovulatory discharge of gonadotrophin, but also interrupted that which would otherwise result in persistence of active follicles and of estrogen secretion.

Pfeiffer (1936–1941) reported that in rat hypophyses, the steadily secreting male type is determined by early action of androgen during infancy. It now seems probable that this sex difference actually resides in the hypothalamus. The acyclic *constant-estrous* state which Pfeiffer obtained in female rats by prepubertal testis grafts was irreversible. Luteinization could not be induced by estrogen injection (1936) nor could cycles be established by progesterone therapy (1941). The acyclic *spontaneous persistent-estrous* state in intact females on the other hand, is reversible, as indicated above. Especially interesting in view of Pfeiffer's work, is the report that normal cycles and luteinization can be induced in spontaneously persistent-estrous rats (DA strain) by daily injection of small amounts of testosterone propionate (Marvin, 1948). It appears that in rats during infancy the action of androgen conditions differentiation of the hypothalamic "center" as an intrinsically acyclic mechanism. However, in an intact genetic female or in a male castrated in infancy and implanted with ovaries, the "center" differentiates as an intrinsically cyclic mechanism. Steady states may be induced later by certain environmental or internal factors, yet the cyclic potentiality tends to remain.

Whether only one or several neural centers are concerned in activation of the adenohypophysis cannot be judged from available evidence. Nor would we make the doubtful claim that all functions of the hypophysis depend on neural stimulation, even all gonadotrophic functions. There seems no question, however, that a revision of current views of gonadotrophin secretion is distinctly called for.

SUMMARY AND CONCLUSIONS

In the preceding article it was shown that Dibenamine or atropine will block the mechanism which leads to release of LH from the adenohypophysis after estrogen injection. Since these drugs do not interfere with the glandular discharge of the hormone nor with its action on the ovary, the conclusion was reached that estrogen effects LH release at least partially by way of the nervous system, presumably the hypothalamus.

The existence in the cyclic rat of a similar mechanism and the chronologic relationship of this to the cycle are demonstrated in the present report, which concerns the results of injections of Dibenamine or of atropine at various hours of the day of proestrus.

Excepting preliminary experiments, the animals used were 68 4-day cyclic rats belonging to a strain in which ovulation normally occurs between 1 and 2:30 A.M. in the early morning after proestrus. When Dibenamine was injected during proestrus at 2 P.M. or earlier, ovulation was prevented or significantly retarded in most cases. Injection of atropine at these hours uniformly prevented preovulatory swelling or ovulation. Associated with these blocking effects of either drug was the failure both of the ovarian interstitial tissue to undergo the usual cholesterol depletion and of the corpora lutea of the preceding cycle to store cholesterol. The uteri usually remained distended the day after injection. In most cases the vaginal smear sequence was retarded significantly.

When either drug was injected at 4 P.M. during proestrus, ovulation proceeded without detectable interference apart from retardation in a few rats treated with Dibenamine. In the majority of cases the only suggestion of an effect upon LH release was an apparent delay of luteal cholesterol storage.

It is concluded that in this strain of rats, under our colony conditions, neurohumoral stimulation of the adenohypophysis occurs during proestrus between 2 and 4 P.M., 10 to 12 hours before ovulation. The demonstration that this chronologically limited neurohumoral stimulus is essential to adequate release of LH, clearly offers a means by which various features of the polyestrous cycle may be synchronized with environmental rhythms.

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VARIABLES AFFECTING THE ASSAY OF TESTOSTERONE PROPIONATE USING THE SEMINAL VESICLE RESPONSE OF THE JUVENILE CASTRATED MALE RAT

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INTRODUCTION

THE USE of the accessory sex organs as the criterion of response for the biological assay of androgens has been described by Korenchevsky 1932, Korenchevsky et al. 1932, Callow and Deansley 1935, Bulbring and Burn 1935, Deansley 1938, Greene and Burrill 1940, 1941, Hays and Mathieson 1945, and Mathieson and Hays 1945.

The procedure as outlined by the latter authors has been found a practical procedure for the assay of commercial preparations of testosterone propionate. However, the error of the method in the experience of this laboratory appeared relatively large and a study of some of the variables affecting the response was deemed advisable in order to determine whether any improvement in the precision of the assay could be obtained consistent with economy of materials, time and effort.

METHODS

Immature rats of Wistar strain bred and raised in this laboratory were used. They were castrated at 28 to 29 days of age and within the weight range of 40 to 60 grams. The following technique was used for the removal and weighing of the seminal vesicles. After killing the rats with chloroform and exposing the internal sex organs, the seminal vesicles were stripped of their fibrous capsules and removed with fine forceps directly under a binocular dissecting microscope. The seminal vesicles were then washed in saline, excess fluid taken up by blotting briefly with filter paper and weighed on a Roller Smith balance sensitive to 0.2 mg. In the case of the assays shown in table 1, assays 7 to 12, the technique was as outlined by Mathieson and Hays (1945). Mazola oil (a brand of corn oil) was used as a diluent for the doses. Crystalline testosterone propionate U.S.P. XIII was used as a standard of reference, and the tests were carried out on market samples of testosterone propionate or on known dilutions of the reference standard. In the case of assays two weeks were allowed after castration before using the rats and three dosage levels of standard and sample were employed, the rats being distributed for dosing on the basis of equal body weight at the time

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of castration. The doses were injected intramuscularly with a 1 cc. tuberculin syringe carrying a 26 gauge $\frac{5}{8}$ inch needle, and subcutaneously with a 24 gauge $1\frac{1}{4}$ inch needle. Precautions were taken in the injections to prevent leakage of the doses by inserting the needles full length. The results were subjected to analysis of variance as outlined by Bliss and Marks (1939), the

TABLE 1. THE EFFECT OF THE VOLUME OF THE INJECTION AND METHOD OF ADMINISTRATION ON THE SLOPE OF THE REGRESSION LINE, VARIANCE AND CONFIDENCE LIMITS OF THE ASSAY

Assay	No. of Rats	Slope b	Variance s^2	Confidence Limits in Per Cent Sm. $\times 1.96$
	0.1 cc. Subcutaneously			
1	42	13.95	17.86	65.4 to 153.0
2	48	7.97	18.12	49.7 to 201.2
3	48	8.30	18.50	49.9 to 200.3
4	48	8.06	20.20	43.9 to 227.7
5	48	9.05	16.60	53.9 to 185.7
6	48	9.37	20.24	51.4 to 194.2
Mean		9.36	18.61	
	0.2 cc. Subcutaneously			
7	48	9.25	13.97	59.0 to 169.0
8	48	9.30	7.74	67.7 to 147.8
9	48	14.05	13.13	71.4 to 140.0
10	48	8.25	8.62	60.7 to 164.2
11	48	6.93	9.28	55.0 to 181.9
12	48	7.04	8.61	58.0 to 172.4
Mean		9.14	10.23	
	0.1 cc. $\times 2$ Intramuscularly			
13	48	12.09	3.93	80.7 to 123.9
14	48	11.54	3.04	81.9 to 122.0
15	48	12.45	1.60	87.6 to 114.2
16	48	12.62	3.12	83.3 to 120.0
17	48	10.38	3.01	80.3 to 124.6
18	48	12.96	5.08	79.7 to 125.4
Mean		12.01	3.30	

values for the slope of the regression line and the variance of the assay being used as indicators of the estimate of the precision of the assay.

RESULTS

Table 1 shows the results obtained in the assay of market samples of testosterone propionate employing first the injection technique as outlined by Mathieson and Hays (1945) of using 0.1 cc. subcutaneously, secondly, of using 0.2 cc. subcutaneously and finally of using intramuscular injections and giving the dose in a volume of 0.1 cc. into the muscle of each hind leg (0.1 cc. $\times 2$ intramuscularly). It is seen that increasing the volume of the injection from 0.1 cc. to 0.2 cc. subcutaneously tends to decrease the variance of the assay (column 4) and the precision as reflected in the calculated confidence limits (column 5). When the injections are given intramuscularly the slope of the regression line is within the same range as for subcutaneous

injection, but the variance of the assay is markedly decreased and the estimate of the confidence limits reduced to values which are considered reasonable for most biological assays. Thus it is seen that intramuscular administration of the doses improves the precision of the assay of testosterone propionate.

In order to obtain a direct comparison of the two methods of administration the tests shown in table 2 were performed using known dilutions of the testosterone propionate reference standard. In the first group of assays (columns 2 and 3) the results obtained using 24 rats (8 per dosage level) and 0.1 cc. subcutaneously were compared with the results obtained using 24 rats (8 per dosage level) and 0.1 cc.×2 intramuscularly (columns 4 and 5). The dosage levels used for

TABLE 2. THE EFFECT OF SUBCUTANEOUS AND INTRAMUSCULAR ADMINISTRATION ON THE SLOPES, VARIANCES AND SENSITIVITIES

Assay	Slope b	Variance s^2	Slope b	Variance s^2	Ratio in per cent $\frac{\text{Intramuscular}}{\text{Subcutaneous}}$	Confidence Limits of Ratio in per cent Sm. ×1.96
	0.1 cc. Subcutaneously		0.1 cc. ×2 Intramuscularly			
1	15.44	12.21	10.96	3.06	36.7	28.3 to 49.0
2	13.95	24.51	12.79	4.58	19.3	13.2 to 28.2
3	15.94	17.12	13.45	5.00	28.3	21.0 to 38.1
4	15.44	18.11	13.28	2.09	33.9	25.2 to 45.6
Mean	15.20	18.00	12.62	3.63	30.1	25.7 to 35.2
	0.1 cc. ×2 Subcutaneously		0.1 cc. ×2 Intramuscularly			
1	13.12	8.66	11.79	1.84	55.4	41.3 to 74.3
2	10.79	7.62	10.29	4.12	62.5	42.5 to 91.9
3	9.13	6.40	12.62	1.31	54.7	41.1 to 72.8
4	15.45	8.29	11.46	2.26	47.1	36.5 to 60.8
Mean	12.12	7.75	11.54	3.38	53.2	45.8 to 61.7

the subcutaneous injections were 0.2 mgm., 0.4 mgm. and 0.8 mgm. respectively, while the dosage levels for the intramuscular injections were 0.05 mgm., 0.10 mgm. and 0.20 mgm. respectively. It is seen that the slopes of the regression lines for the two routes of administration are within the same range, but the variance of the test is definitely higher (mean 18.00) in the case of the subcutaneous injections than in the case of the intramuscular administration (mean 3.63). In column 6, the ratios of the activity of the intramuscular injections to the subcutaneous injections are shown. It is seen that the rats are considerably less sensitive to the subcutaneous injection (mean 30 per cent) or it requires approximately three times as much testosterone propionate subcutaneously to produce a comparable response intramuscularly. The estimate of the confidence limits of the ratio is shown in the last column.

In order to determine if the division of the doses subcutaneously would produce results comparable to the intramuscular administration the tests shown in the second group of assays in table 2 were performed. The same number of rats per assay and the same dosage

levels of testosterone propionate were used as in the first group. In the case of the subcutaneous administration the injections were made in amounts of 0.1 cc. into the loose subcutaneous tissue on opposite sides of the rat. In this group of assays the variances of the tests (column 3) are lower (mean 7.75) using 0.1 cc.×2 subcutaneously than the variance (mean 18.00) using 0.1 cc. subcutaneously in the first group. The ratios of the activity (mean 53.2%) shown in column 6 of intramuscular to subcutaneous administration indicate that the division of the doses subcutaneously increases the sensitivity, however, it required approximately twice as much testosterone propionate at 0.1 cc.×2 subcutaneously as it did at 0.1 cc.×2 intramuscularly to produce comparable responses.

TABLE 3. THE EFFECT OF THE METHOD OF ADMINISTRATION ON THE PRECISION OF THE ASSAY

Method of Administration	No. of Assays	$(s/b)^2$	No. of rats for Comparable precision
0.1 cc.×2 Intramuscularly	6	.023	1.0
0.1 cc.×2 Subcutaneously	4	.051	2.2
0.2 cc. Subcutaneously	6	.123	5.3
0.1 cc. Subcutaneously	6	.212	9.3

Table 3 is a summary of the results obtained with the different methods of injection employed using the ratio of the value of the slope to the variance as an indicator of the estimate of precision. Since the method using 0.1 cc.×2 intramuscularly gave the lowest value for $(s/b)^2$ (column 3) this was used as a basis of comparison and was given the value 1.0 (column 4), while the remaining methods of injection are expressed in terms of this indicating the number of rats required in an assay for comparable precision. These results show that in the intramuscular administration of the doses there is considerable economy of rats and the division of the doses subcutaneously is quite superior to single injections with respect to the number of rats required for comparable precision.

The influence of the time of autopsy on the seminal vesicle response to testosterone propionate administered intramuscularly (0.1 cc.×2) is shown in figure 1. Each point on the graph represents the mean seminal vesicle weight of 10 rats killed 24, 48, 72 and 96 hours after injection at the dosage level of 0.05 mgm., 0.10 mgm. and 0.20 mgm. respectively. It is seen that the maximum response is obtained at 72 hours after injection on the two lower dosage levels, while at the higher dosage level the response is maintained until 96 hours after injection. These results are similar to those reported by Hays and Mathieson (1945) indicating that the time relationship of the response for the intramuscular injection of the doses are the same as those for the subcutaneous injection of the doses.

Hooker (1942) has shown using male rats castrated at birth that the sensitivity of such rats to testosterone increased markedly at 40, 50 and 60 days of age, this being the age when the accessory sex glands normally undergo puberal changes. Since it is difficult to obtain a sufficient number of immature rats for castration at one time to perform an assay, it is customary to assemble rats at various post castration ages as recommended by Hays and Mathieson (1945). The effect of testosterone propionate on the seminal vesicle response of the juvenile castrated rats over the above age range was not well covered by the above workers and it was considered advisable to de-

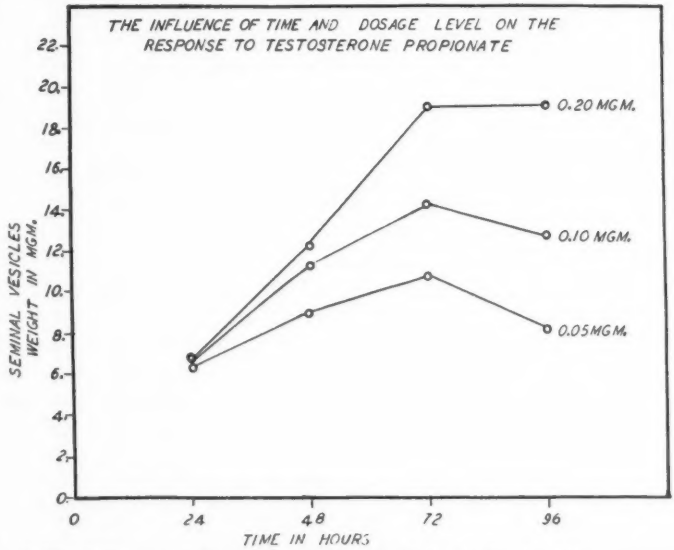

FIG. 1. The influence of the time of autopsy and dosage level on the response of testosterone propionate administered intramuscularly.

termine if the alterations in sensitivity reported by Hooker (1942) might account for some of the variations in response encountered in assembling rats for assay of the various post castration ages.

The following experiment was designed to determine this point. Fifteen rats were castrated and 10 of these were injected immediately with 0.2 mg. of testosterone propionate (0.1 cc.×2 intramuscularly) and 5 were kept as controls. Seventy-two hours later the group was killed and the seminal vesicle removed and weighed. In the same manner other groups of 15 rats were castrated, except before injection 7, 14, 21, etc. days elapsed after castration. In this way the seminal vesicle response to 0.2 mgm. of testosterone propionate and untreated controls were followed over the age range from 28 to 98 days. The results are shown in figure 2. It is seen that the rats tend to be more

sensitive to testosterone propionate during the first two weeks after castration, but after this time the response is relatively uniform. Applying the X^2 tests to the mean of all the points it is seen that they do not deviate significantly from a straight line $X^2=0.98$. At 10 degrees of freedom $X^2=18.03$ p at 0.05. However, when the first two points are eliminated the fit to a straight line is considerably improved and does not differ from the results obtained for the untreated con-

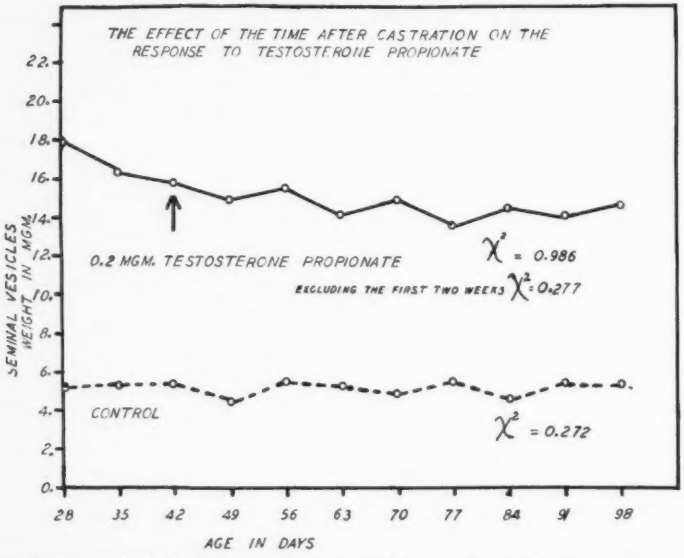

FIG. 2. The effect of the time after castration on the response to 0.2 mgm. of testosterone propionate administered intramuscularly—solid line and on untreated controls—broken line.

trols. No definite break or trend is indicated after the first two weeks post castration. These results indicate that, except for the first two weeks after castration, the sensitivity of rats castrated at 28 days of age to testosterone propionate does not change significantly and the use of rats for assay of various post castration ages does not affect the variations encountered in the response.

DISCUSSION

The assay of testosterone propionate using the seminal vesicle response of the juvenile castrated male rat has been found to be a practical procedure for checking the potency of commercial samples of this androgen. The advantage of using intramuscular injection of the doses is shown in tables 1, 2 and 3. A better utilization of the testosterone propionate is indicated from the lower value of the variance and the

greater sensitivity in the intramuscular injections than in the subcutaneous injections, table 2. Leathem (1948) has shown recently that the response to testosterone propionate injected subcutaneously was not influenced by the volume of oil and that the subcutaneous and intramuscular routes of administration increased accessory sexual organ weight equally well. The difference between these results and those reported here may be due to the use by Leathem (1948) of uncastrated immature rats and including the weight of the coagulating glands with the weight of the seminal vesicles. Diluents other than Mazola oil for the dosage levels were not tried and this may be a factor in the response as shown by Crafts (1942) in the seminal vesicle response of mice to sesame oil and by Albrieux and Prego (1943) on the activity of estrone in various oils as diluents.

The results obtained in the assays shown in table 1 were subjected to factorial analysis in order to determine if linear dosage response relationships existed over the dosage range used. The variance ratio value for the slope of the dosage response line was highly significant in all the assays and in none of the assays was there any indication from the variance ratio value of lack of parallelism between the slope of the regression line of the standard and unknown. The variance ratio value also showed no significant degree of curvature or opposed curvature in the regression lines of any of the assays.

The time relationships of the response (figure 1) to intramuscular administration are similar to those reported by Hays and Mathieson (1945) for subcutaneous administration. The maximum response is obtained at approximately 72 hours after injection and there is a better differentiation of the response at this time than at 48 hours after injection as recommended by Greene and Burrill (1941). The assembling of rats of various castration ages is justified in accordance with the results shown in figure 2. It is evident that the sensitivity of the rats castrated at 28 days of age does not change significantly as reported by Hooker (1942) for rats castrated at birth.

This method of assay was not found applicable for checking the potency of commercial samples of methyl testosterone or testosterone on account of relatively large amounts required to produce a response (5 to 10 mgm.) and the low solubility of these products in oil presented difficulties in obtaining convenient volumes for injection (unpublished results.).

SUMMARY

In a study of the variables affecting the assay of testosterone propionate using the seminal vesicle response of the juvenile castrated rat it was found that the precision and sensitivity of the assay could be improved some by the division of the doses administered subcutaneously and considerably by intramuscular administration of the doses. Except for the first two weeks after castration, the sensi-

tivity of rats castrated at 28 days of age to testosterone propionate does not change significantly and the variations in response of the seminal vesicles are not influenced by assembling rats of various castration ages.

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THE ADMINISTRATION OF ADRENOCORTICOTROPHIC HORMONE TO NORMAL HUMAN SUBJECTS. THE EFFECT OF THE LEUCOCYTES IN THE BLOOD AND ON CIRCULATING ANTIBODY LEVELS

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THE FIRST suggestion that the adrenals might be concerned in the general defence reaction of the body followed observations that these organs sometimes showed marked pathological changes during acute infective and toxic episodes. Several studies were made in which the effect of adrenalectomy on antibody titres was investigated. Reports were contradictory. Thus Hektoen and Curtis (1915) found that the level of circulating antibodies in dogs was not altered by bilateral operation and Gates (1918) reported that the rate of antibody formation by rabbits was unaffected by previous partial adrenalectomy. Jaffe and Marine (1924), on the other hand, obtained higher titres after bilateral adrenalectomy when the animals had been immunized three weeks after operation, but noted that at six weeks there was no difference in the response between operated and unoperated rabbits. Their results were in direct contradiction to those of Perla and Marmorston-Cottesman (1928) who noted decreased rates of antibody production in rats injected during the five weeks following operation, this decrease being most marked in the first week.

As the chemistry and physiology of the adrenal cortex were made clearer it became possible to work on animals under more nearly physiological conditions. Fox and Whitehead (1936) were the first to try the effect of injecting adrenal cortical extract into experimental animals. They reported an increased rate of production of antibodies following injection of the hormone but found that control animals subsequently attained much the same titres.

These experiments were repeated and extended by Chase, White and Dougherty (1946). Using cortical extracts, synthetic cortical hormones and adrenocorticotrophin (ACTH), they have worked both with normal animals and with adrenalectomised animals maintained on desoxycorticosterone acetate (DCA). They have produced evi-

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dence, not only that the cortical hormones increase antibody levels when given during the immunisation period, but also that they can effect an immediate release of stored antibody into the circulation. Recently an attempt was made by Eisen *et al.* (1947) to measure the first of these effects more precisely by using an accurate chemical method of antibody determination. They were unable to show that the production of antibody was affected by the injection of cortical hormones or that it was dependant on the presence of the adrenal. They did, however, partially confirm the findings of Chase, White and Dougherty in that they sometimes noted transient increases of antibody nitrogen immediately after injection of cortical extracts.

The experiments described below were designed, to elicit this latter effect in human subjects following stimulation of the adrenal cortex by ACTH. Staphylococcal toxoid was chosen for the immunising agent, as the estimation of anti-haemolysin is quick and can be carried to a finer end-point than titrations involving agglutinations. The differential leucocyte counts which are also reported were a partial check on the activity of the hormone preparation, one of the results of adrenal cortex stimulation being a decrease in the numbers of circulating lymphocytes (Dougherty and White, 1944) and eosinophils (Forsham *et al.* 1948).

TECHNICAL METHODS

Antibody to staphylococcus alpha-haemolysin. The potency of the toxin was measured initially against a sample of international standard anti-serum and thereafter occasionally checked against a sub-standard serum. In titrating the unknown sera the toxin was diluted with 0.85% NaCl so that 1 ml. contained either 1 L.H. unit or $\frac{1}{5}$ L.H. unit. The latter dilution was used when the sera were of low potency and of insufficient quantity to allow accurate titration at the higher level. In any one experiment all the sera were titrated simultaneously, using the same batch of diluted toxin. After heating for 40 minutes at 56°C. to inactivate complement, the sera were incubated with a known volume of a rabbit red cell suspension to absorb the red cell agglutinins and final dilutions were then made with saline from the supernatant fluid. 1 ml. of each dilution was incubated with 1 ml. of toxin for 1 hour at 37°C. and 0.5 ml. of a 5% suspension of washed rabbit cells added to each tube. After a further 60 minutes incubation the tubes were kept at about 4°C. overnight. They were then centrifuged and the end-point taken as mid-way between the dilution of serum showing no haemolysis and that showing trace or partial haemolysis.

Agglutinins to the typhoid "H" antigen. The antigen was a formalized suspension of E. typhosa "Buzzard" containing approximately 400 million organisms per ml. Doubling dilutions of sera in saline were made and 1 ml. of each dilution, together with 1 ml. of antigen suspension, was incubated at 56°C. After 6 hours the end-point was taken as the highest serum dilution showing complete agglutination.

Total leucocyte counts and eosinophil counts were made according to the method described by Randolph (1944), using a water-propylene glycol mixture as the diluting fluid. For the total leucocytes, the cells contained in the

four large corner squares in each of four or eight chambers were counted. For the eosinophils the cells within the entire ruled area of at least 10 chambers were counted.

Smears for the *differential cell counts* were made on coverslips and stained by Wright's method. Areas extending outwards from the tail of the smear were chosen, having regard to even distribution of the leucocytes, lack of fragmentation and thinness of the film. The number of cells counted from each sample is given in the tables of results.

Haematocrits were measured in Wintrobe tubes after spinning for 45 minutes at *ca.* 3000 r.p.m. Two tubes were filled and the average of the two readings is reported. The speed of centrifugation was checked occasionally by reading the haematocrits after 30, 45 and 60 minutes.

Plasma and serum protein values were determined by the method of Jacobsen and Lindstrøm-Lang (1940), using the Philips, van Slyke *et al.* (1945) nomogram for the conversion of R.D. to gms. protein/100 ml. The standards used ($CuSO_4$ solutions) were also taken from the latter communication and were checked by direct weighing in a 25 ml. capacity specific gravity bottle. The density of whole blood was measured in some experiments and packed cell volumes calculated from this usually agreed with those determined by direct measurement.

Glycogenic corticoids and 11-oxy steroids in the urine were measured by Dr. E. H. Venning and are reported in a separate communication (1948). Urinary corticoids were determined by bioassay according to the method of Venning *et al.* (1946) and 11-oxy steroids by the method of Talbot *et al.* (1945).

SUBJECTS

The subjects of the experiments were healthy normal adults who had been inoculated with commercial preparations of typhoid vaccine and staphylococcal toxoid.

Subject "H"—female, aged 29 years—received combined T.A.B. vaccine and tetanus toxoid in 1941 and a routine course of staphylococcal toxoid (0.05 ml. increasing to 1.0 ml.) during March 1947. This was followed by a single injection of 2.0 ml. staphylococcal toxoid on June 16th. The highest values for anti-haemolysin were found in April 1947 and were between 11 and 12 Provisional International Units (P.I.U.) per ml. serum.

Subject "Rs"—male, aged 39 years—received combined T.A.B. vaccine and tetanus toxoid in 1944 followed by several "booster" injections, the last being given in July 1947. He received the same routine course of staphylococcal toxoid as subject H, in May 1947. The highest recorded values for anti-haemolysin were found in June 1947 to be about 10 P.I.U./ml.

Subject "Ha"—male, aged 32 years—received combined T.A.B. vaccine and tetanus toxoid prior to 1945 with the last injection in the winter of that year, and a course of staphylococcal toxoid during September and the beginning of October 1947. After a preliminary injection of 0.25 ml. four injections of 0.5 ml. and one of 1.0 ml. were given at weekly intervals. Measurements of the anti-haemolysin values for the serum of this subject were not made until shortly before the start of the experiment.

Blood samples for control leucocyte counts were also taken from three other individuals. Subject "Gr" was a male, aged 31 years, and Subject "V," a female aged 29 years. Subject "Go" was an apparently healthy

airman aged 24 years. He was later found to have an impaired glucose tolerance but was otherwise normal.

EXPERIMENTAL MATERIAL

Adrenocorticotrophin was supplied by the Armour Laboratories. Two preparations were used, both of which had been made from hog pituitary glands by a modification of the method of Sayers *et al.* (1943). Batch 32D, which was used in experiments 1 to 4, contained 0.1 unit pitressin/mg. but Batch 37KE, which was used in experiment 5, contained only a trace of this material. Other pituitary hormones were absent or present only in negligible quantities. Both batches were assayed by the Armour Laboratories against their standard preparation LA-1-A. Batch 32D was found to have 70%, and Batch 37KE 50%, of the adrenocorticotrophic activity of this preparation.

The material supplied was a powder and was dissolved in saline prior to intramuscular injection. Batch 37KE had previously been sterilised and Batch 32D was sterilised by boiling for 15 minutes immediately after the solution had been made. The dissolved material was occasionally stored overnight before using and was then kept frozen at −20°C.

RESULTS

Adrenocorticotrophin was given to normal subjects on five occasions. In addition to these experiments various control leucocyte counts were made at different times of day. As the preparation of

TABLE 1. THE DIURNAL VARIATION IN LEUCOCYTE COUNTS UNDER NORMAL CONDITIONS AND AFTER THE INJECTION OF PITRESSIN

Subject & Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of Cells Examined
		Total	Neutrophil	Lymphocyte	Eosinophil (Direct)	Monocyte	
"V" 17/ 9/47	10:00 A.M.	5,910	3,460	1,790	188	420	2,000
	2:30 P.M.	6,540	3,870	2,100	195	510	2,000
	5:00 P.M.	7,320	4,300	2,420	245	600	2,000
	8:00 P.M.	8,490	5,600	2,380	238	476	2,000
"H" 22/ 9/47	10:00 A.M.	8,930	5,370	2,640	164	652	2,000
	1:30 P.M.	9,550	6,280	2,460	153	572	2,000
	4:45 P.M.	11,200	6,820	3,270	150	851	2,000
	8:45 P.M.	13,540	8,440	3,840	250	866	2,000
"Gr" 15/10/47	10:00 A.M.	5,560	2,420	2,650	89	378	1,000
	4:30 P.M.	6,410	3,250	2,550	123	500	1,000
	8:00 P.M.	6,905	3,220	3,040	144	456	1,000
"H" 8/11/47	10:00 A.M.	10,010	5,200	3,750	Not done	900	2,000
	12:15 P.M.	9,900	6,170	2,690	Not done	841	2,000
	2:45 P.M.	10,120	6,320	3,028	Not done	668	2,000
"Go" 14/ 4/48	10:00 A.M.	5,530	3,750	1,140	122	514	2,000
	12 noon	6,280	3,750	1,800	137	559	2,000
	4:00 P.M.	6,960	4,130	1,930	188	689	2,000
	8:00 P.M.	7,630	5,370	1,610	178	466	2,000
20 units pitressin, intramuscularly—5 units at 10:05 A.M., 12 noon, 2:00 P.M., 3:00 P.M.							
"H" 10/ 9/47	10:00 A.M.	9,420	6,360	2,050	109	791	1,000
	5:00 P.M.	13,620	9,940	2,550	75	926	1,000
20 units pitressin, intramuscularly—10 units at 10:05 A.M., 11:00 A.M.							
"H" 15/ 9/47	10:00 A.M.	10,480	7,200	2,350	141	776	1,000
	12:30 P.M.	11,300	8,500	1,840	53	870	1,000
	4:00 P.M.	12,970	9,090	2,780	94	986	1,000

hormone initially available (32D) contained appreciable amounts of posterior pituitary contaminant, some counts were also made on one subject following the injection of 20 units of pitressin. 200 mgs. of the corticotrophin Batch 32D assayed as containing this amount of pitressin.

All these control findings are summarised in Table 1.

In general the findings confirmed those of Elmadjian and Pincus (1946) with regard to the lymphocytes and those of Rud (1947) with regard to the eosinophils. The latter author has drawn attention to the marked variation which may occur even from minute to minute in the absolute eosinophil count. The range of normal levels noted by

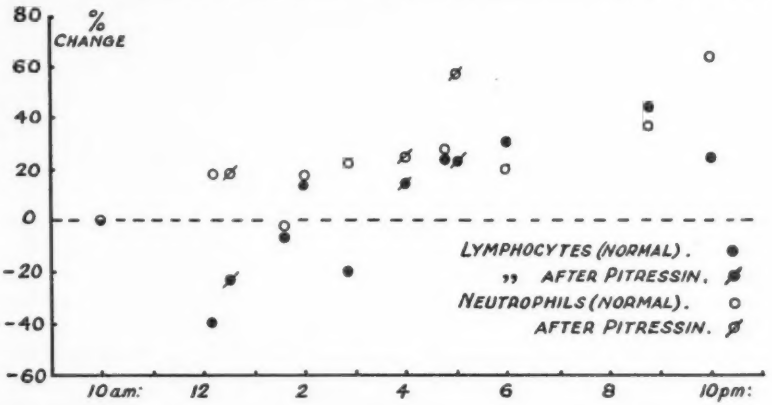

FIG. 1. Diurnal variations in lymphocyte and neutrophil counts in a female subject (H) on five separate control days. (See also Tables 1 and 6).

Percentage changes plotted in this and subsequent figures have been calculated from values obtained at or about 10 A.M.

him was quite similar to that shown in Table 1, although the variations in his subjects were rather more marked.

This variability of the eosinophil count makes it difficult to decide whether the fall noted after giving pitressin to subject "H" should be attributed to the effect of the hormone or not. If on these two occasions stimulation of the adrenal occurred as part of the reaction to pitressin it was not of the same degree as that induced on other occasions by injection of corticotrophin.

In Figure 1 the neutrophil and lymphocyte changes for five control days in Subject "H" are charted.

The early fall and later steady rise in the lymphocytes reported by Elamadjian and Pincus is well shown here. With regard to the monocytes, it can only be stated that the variations are of the same degree as those noted later in the corticotrophin experiments. It is unlikely that sufficient cells were examined to establish the significance of these variations.

TABLE 2. *Experiment 1.* EFFECT OF 40 MG. ACTH ON THE BLOOD PICTURE OF A FEMALE SUBJECT (H)

Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of cells examined	Haematocrit Vol. %	Serum proteins gms./100 ml.	Antihaemolysin PIU/ml.[1] serum	Agglutinin Titer
		Total	Neutrophil	Lymphocyte	Eosinophil (Direct)	Monocyte					
8/7/47	10:20 A.M.[2]	8,250	4,570	2,900	115	611	1,000	46.4	7.3	6.0	1/40
8/7/47	11:25 A.M.	7,460	4,400	2,570	75	527	1,000	43.9	7.0	5.7	1/40
8/7/47	12:40 P.M.	12,240	8,940	2,780	26	404	1,500	44.7	7.3	6.0	1/40
8/7/47	2:25 P.M.	11,720	9,550	1,680	12	390	1,500	44.7	7.2	5.8	1/20
8/7/47	5:00 P.M.	12,870	9,570	2,670	17	579	1,000	45.3	7.5	7.0	1/20
8/7/47	8:30 P.M.	13,120	8,470	3,700	129	700	1,000	45.2	7.2	5.4	not done
9/7/47	11:20 A.M.	9,390	6,350	2,360	81	730	1,000	43.4	7.0	5.1	1/40

[1] PIU indicates "provisional international unit."
[2] ACTH (32D) =28 mg. Armour standard LA-1-A given immediately after withdrawl of this sample.

TABLE 3. *Experiment 2.* EFFECT OF 100 MG. ACTH ON THE BLOOD PICTURE OF A MALE SUBJECT (Rs)

Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of cells examined	Haemocrit Vol. %	Plasma proteins gms./100 ml.	Antihaemolysin PIU/ml. serum	Agglutinin Titre
		Total	Neutrophil	Lymphocyte	Eosinophil (Direct)	Monocyte					
1/9/47	1:00 P.M.	4,780	2,460	1,800	102	344	2,000	not done	not done	5.7	1/80
2/9/47	10:00 P.M.[1]	6,160	3,030	2,290	149	570	1,000	41.8	7.4	6.2	1/80
2/9/47	12 noon	5,410	3,840	1,090	57	303	1,000	39.7	7.0	5.7	1/80
2/9/47	1:50 P.M.	10,220	9,110	730	18	290	1,000	39.3	7.1	6.0	1/160
2/9/47	4:00 P.M.	8,730	7,730	770	6	183	1,000	39.2	7.3	6.2	1/80
2/9/47	6:00 P.M.	9,410	7,740	1,280	16	352	1,000	40.1	7.4	5.5	1/80
2/9/47	8:10 P.M.	9,800	7,370	2,050	44	314	1,000	41.2	7.4	5.2	1/80
3/9/47	6:00 P.M.	7,000	4,020	2,070	148	640	1,000	not done	not done	5.7	1/80

[1] ACTH (32D) =70 mg. Armour standard LA-1-A 50 mg. given immediately after withdrawal of this sample, the remainder one hour later.

Experiments with Adrenocorticotrophin

Experiments 1 to 3. In these an attempt was made to induce a sudden and pronounced increase in adrenal cortical activity. The quantity of hormone that could be injected at one time was limited by the pitressin content of the preparation. To minimize the effect of this the larger doses of corticotrophin were divided and injected over periods of 1–2 hours. In spite of this, one subject ("Rs", experiment 2) showed a very marked reaction, presumably due to the posterior pituitary contaminant. After receiving two injections of 50 mgs. corticotrophin, separated by an interval of 60 minutes, his resting blood pressure rose in 30 minutes to 180/120 and his pulse rate, normally around 80, fell to 65. He had diarrhoea and marked abdominal cramps. One hour after the injection his blood pressure had fallen to 134/88 and an hour later he had apparently recovered. In this experiment, then, one cannot exclude the possibility that the reaction to pitressin in itself produced a sufficient "stressful" situation to stimulate the adrenal cortex. In experiment 1 where 40 mgs. of the same preparation were used and in experiment 3 with 200 mgs. no serious reaction occurred. It was noted, however, that the pallor which occurred following the injections was of much longer duration and more intense than that following the injection of similar or larger quantities of purified posterior pituitary hormone.

The results of these experiments are shown in Tables 2–4.

The findings in these three experiments were very similar and are illustrated by Figure 2, where the results from experiment 3 are charted.

In the white cell counts the eosinophils showed the most marked change. The fall seen in experiment 3 occurred in all experiments and is apparently related in both degree and duration to the quantity of hormone administered. While the number of lymphocytes also decreased in this and other experiments, this was not more pronounced with the larger doses of corticotrophin. These cells always returned more quickly to normal than the eosinophils. The rise in the neutrophil count is similar to that produced by the injection of other foreign proteins. Like the lymphocyte response, it is variable in degree and duration.

The serum protein and haematocrit values are of interest. Although the heamatocrit fell by 11%, indicating an increase in plasma volume, the serum protein values were not decreased. They were on the contrary slightly raised. In the first two experiments also, the haematocrits fell, but not more than is usual in the course of the day.

Values for anti-haemolysin are also shown in Figure 2. They are decreased slightly in parallel with the haematocrit, but the changes noted are of doubtful significance. In the first experiment with 40 mgs. corticotrophin there was a definite but slight increase 6½ hours after the injection (Table 2), whereas in the 100 mg. experiment

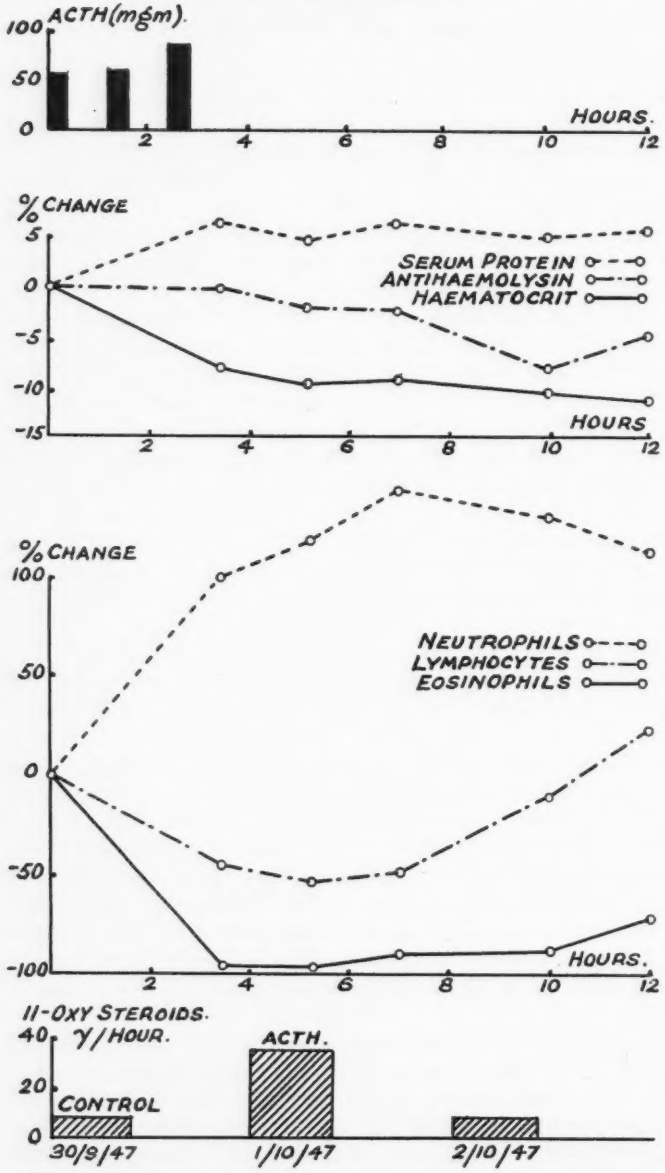

FIG. 2. Experiment 3. Suject H. The effect of 200 mg. ACTH (see Table 4).

(Table 3), a fall of about the same extent was noted at 10 hours. In these three experiments agglutinins to the typhoid "H"" antigen were also titrated and showed no marked change. In experiment 2 (Table

TABLE 4. *Experiment 3.* EFFECT OF 200 MG. ACTH ON THE BLOOD PICTURE OF A FEMALE SUBJECT (H)

Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of cells examined	Haemo-crit Vol. %	Serum proteins gms./100 ml.	Antihae-molysin PIU/ml. serum	Agglutinin Titre
		Total	Neutro-phil	Lympho-cyte	Eosino-phil (Direct)	Mono-cyte					
1/10/47	9:30 A.M.[1]	8,000	5,130	2,240	103	464	2,000	46.3	7.4	4.2	1/30
1/10/47	1:15 P.M.	12,150	10,370	1,230	6	498	2,000	42.6	7.9	4.2	Not done
1/10/47	3:00 P.M.	12,700	11,180	1,060	4	413	2,000	41.9	7.8	4.1	1/30
1/10/47	4:50 P.M.	14,370	12,620	1,106	11	604	2,000	41.8	7.9	4.1	1/30
1/10/47	7:45 P.M.	14,490	11,780	2,010	12	630	2,000	41.4	7.8	3.9	1/30
1/10/47	9:45 P.M.	14,680	10,850	2,760	28	984	2,000	41.0	7.9	4.0	Not done
2/10/47	3:00 P.M.	9,110	Not done	Not done	114	Not done	Not done	42.7	7.1	4.0	Not done

[1] ACTH (32D) ≡ 140 mg. Armour standard LA-1-A 55 mg. given at 9:45 A.M. 60 mg. at 11:00 A.M. and 85 mg. at 12 noon.

TABLE 5. *Experiment 4.* EFFECT OF 200 MG. ACTH ON THE BLOOD PICTURE OF A MALE SUBJECT (Ha)

Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of cells examined	Haemato-crit Vol. %	Plasma proteins gms./100 ml.	Antihae-molysin PIU/ml. serum	Agglutinin Titre
		Total	Neutro-phil	Lympho-cyte	Eosino-phil (Direct)	Mono-cyte					
18/11/47	10:00 A.M.	6,350	3,683	1,600	117	871	2,000	47.8	7.4	4.9	Not done
18/11/47	2:00 P.M.	6,500	3,650	1,960	140	754	2,000	44.6	7.1	Not done	Not done
18/11/47	6:00 P.M.	6,310	3,430	1,944	110	769	2,000	44.6	7.1	Not done	Not done
19/11/47	10:00 A.M.	9,410	6,630	1,657	139	979	1,000	47.2	7.3	7.0	1/20
20/11/47	10:00 A.M.[1]	15,050	12,900	1,220	6	908	2,000	45.1	7.4	4.6	1/20
20/11/47	12 noon	15,320	12,920	1,270	2	1,118	1,000	42.9	7.1	8.5	Not done
20/11/47	2:00 P.M.	16,420	14,500	1,083	1	821	2,000	44.0	7.5	4.6	Not done
20/11/47	4:00 P.M.	14,920	12,750	1,357	0	805	2,000	42.7	7.4	4.8	1/20
20/11/47	6:00 P.M.	14,870	11,490	2,260	15	1,076	2,000	42.9	7.4	4.6	1/20
21/11/47	10:00 A.M.	8,040	5,320	1,800	100	820	2,000	41.9	6.7	4.5	1/20
22/11/47	10:00 A.M.	6,000	3,336	1,770	100	798	2,000	45.9	7.5	5.2	1/20
24/11/47	10:00 A.M.	6,000	2,934	2,130	142	738	2,000	45.3	7.3	5.4	1/20

[1] ACTH (32D) ≡ 140 mg. Armour standard LA-1-A given in six equal doses. The first was injected at 2:05 P.M. on the 19th November and the remainder at four hourly intervals. The last injection was given immediately after withdrawal of this sample.

3) the titre increased at 3 hours from 1/80 to 1/160, but a change of this degree in agglutination titres is of doubtful validity. A similar change in the opposite direction was noted in experiment 1 (Table 2). In Figure 2 the values for 11-oxy steroids observed in experiment 3 are also charted. These were obtained for three six hour urine collections made between 10 a.m. and 4 p.m. on three consectuive days. In the six hours following the injection of corticotrophin there was a fivefold increase in the excretion of these compounds indicating that adrenal cortical activity was in fact increased. In the first two experiments, biological assay of the urine for three 48 hour periods was carried out and showed no change in the sample covering the experimental day. Presumably the cortical response was so brief that it did not noticeably change the total excretion of corticoids occurring in 48 hours.

Experiments 4–5. Having failed to observe any appreciable rise in antibody levels in the above experiments it seemed possible that a longer period of adrenal stimulation might be necessary to produce this effect.

In experiment 4 (Table 5) six injections at four hourly intervals were given, such that the total quantity of hormone received was 200 mgs. This preparation of the hormone was the same as that used in experiments 1 to 3. It caused slight dizziness and marked pallor in the subject, together with some abdominal cramps. The values obtained for the anti-haemolysin in this experiment are puzzling. Two control sera were obtained and a marked rise (43%) in the anti-haemolysin level was seen to have occurred before injection of the hormone started. No further blood samples were withdrawn until immediately before the last injection. The anti-haemolysin at this time had fallen to the initial control level. Two hours later it had risen 73% above this and after four hours had again returned to the previous level. At first it seemed that the samples drawn at 10 a.m. on the 19th and the 20th might have been inadvertently interchanged and that in fact a rise in antibody titre had been induced by administration of the hormone. There are three things which suggest that this was not a correct hypothesis. In the first place there was an interval of 24 hours between the taking of the two samples, making it extremely unlikely that mis-labelling had occurred. Secondly, if the anti-haemolysin level at 10 a.m. on the 20th was 7.0 units it must have been due to an effect lasting at least four hours, as the previous injection of corticotrophin had been given at 6 a.m. A similar effect should then have been noted not only in the 12 noon serum, which was taken two hours after the last injection, but also in the 2 p.m. sample. Finally, there was no parallel change in the typhoid agglutinin titres.

In view of these inconclusive findings the next experiment was designed along similar lines. By then a new preparation of cortico-

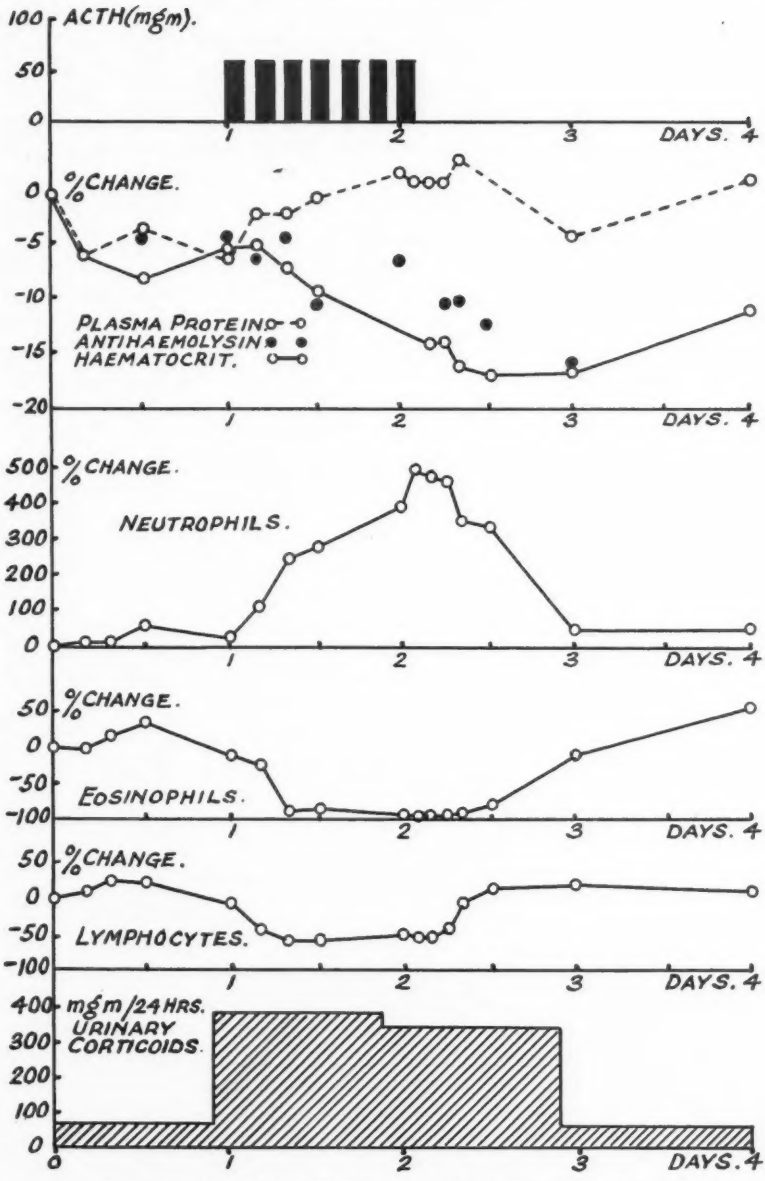

FIG. 3. Experiment 5. Subject H. The effect of 420 mg. ACTH (see Table 6).

trophin (Batch 37KE) had become available. This material had a very low pitressin activity and consequently a larger amount of the hormone could safely be injected at one time. In all 420 mgs. were

TABLE 6. *Experiment 5.* EFFECT OF 420 MG. ACTH ON THE BLOOD PICTURE OF A FEMALE SUBJECT (H)

Date	Time	Leucocyte Counts Per Cubic Millimetre					Number of cells examined	Haematocrit Vol. %	Plasma proteins gms./100 ml.	Antihaemolysin PIU/ml. serum	Agglutinin Titre
		Total	Neutrophil	Lymphocyte	Eosinophil (Direct)	Monocyte					
26/1/48	10:00 A.M.	7,310	4,198	2,318	95	651	1,000	45.7	7.1	4.9	1/60
26/1/48	2:00 P.M.	8,200	4,885	2,632	90	516	1,000	42.8	6.7	Not done	Not done
26/1/48	6:00 P.M.	8,800	5,070	3,010	107	528	1,000	Not done		Not done	Not done
26/1/48	10:00 P.M.	10,550	6,830	2,930	133	632	1,000	42.2	6.9	4.7	Not done
27/1/48	10:00 A.M.[1]	8,130	5,240	2,260	84	508	1,000	43.2	6.7	4.7	Not done
27/1/48	2:00 P.M.	10,980	8,890	1,373	72	604	2,000	43.3	7.0	4.6	1/60
27/1/48	6:00 P.M.	16,030	14,600	897	11	465	2,000	42.7	7.0	4.7	1/60
27/1/48	10:00 P.M.	17,620	15,930	969	10	687	2,000	41.5	7.1	4.4	1/60
28/1/48	10:00 A.M.	22,090	20,240	1,127	5	718	2,000	Not done	7.3	4.6	1/60
28/1/48	12 noon	26,710	24,280	1,032	3	1,363	2,000	Not done	7.2	Not done	1/60
28/1/48	2:00 P.M.	25,830	23,820	1,037	4	943	2,000	39.8	7.2	Not done	Not done
28/1/48	4:00 P.M.	25,740	23,140	1,364	4	1,184	2,000	40.0	7.2	4.4	1/60
28/1/48	6:00 P.M.	22,260	18,470	2,182	8	1,580	2,000	38.9	7.3	4.4	1/60
28/1/48	10:00 P.M.	21,560	17,740	2,717	18	1,272	2,000	38.8	7.2	4.3	1/60
29/1/48	10:00 A.M.	9,830	6,018	2,722	108	953	1,000	38.6	6.9	4.1	1/60
30/1/48	10:00 A.M.	9,940	6,282	2,580	144	875	1,000	40.1	7.2	Not done	Not done

[1] ACTH (37KE) ≡210 mg. Armour standard LA-1-A given in seven equal doses. The first was injected immediately after withdrawal of this sample and the remainder at four hourly intervals. Subsequent blood samples withdrawn immediately before injections. Last injection followed the 10:00 A.M. sample on the 28th January.

given in seven injections at four hourly intervals, When comparing this with the previous experiment it should be noted that this preparation was of lower potency. While 200 mgs. of preparation 32D were equivalent to 140 mgs. of the standard in experiment 4, in experiment 5,420 mgs. of preparation 37KE were equivalent to 210 mgs. of the standard. The findings of the latter experiment are summarised in Table 6 and some of them are charted in Figure 3. Despite the very marked stimulation of the adrenal which occurred, as shown by urinary glycogenic corticoid activity, there was no increase in anti-haemolysin levels at any time, nor was there any change in the typhoid agglutinin titre. The anti-haemolysin levels in fact fell steadily, roughly in parallel with the haematocrit. The plasma proteins, as in other experiments, did not fall below the control values. The suggestion of an active maintenance of the plasma protein levels by the adrenal cortex, implicit in experiment 3 above, is less apparent here than in experiment 4. In experiment 4 (Table 5) the haematocrit was accompanied on the control day by a fall in the plasma proteins. Following corticotrophin the haematocrit indicated a marked increase in plasma volume. The plasma protein levels on the other hand did not show much change either at the end of the injection period or for eight hours thereafter. This implies a release of protein into the plasma either directly as a result of adrenal cortex stimulation, or indirectly following the consequent fluid and electrolyte shifts. The fact that the protein level later fell by 10% before contraction of the plasma volume had occurred and at a time when the effect of the last injection on the adrenals had probably worn off (24 hours after last injection) suggests that a direct mechanism may have been operative.

The cell changes in both these experiments were similar. Eosinophils almost disappeared from the circulation, lymphocytes were reduced, and neutrophils very markedly increased. In experiment 5, in spite of a reduction in lymphocytes of 55%, the neutrophils were sufficiently increased to make the total leucocyte count at one point 26,700 per cu.mm.

DISCUSSION

In these experiments the administration of corticotrophin was not continued for longer than twenty-four hours and no attempt was made to show whether the adrenal cortex played a part in the formation of new antibodies. It has, however, been suggested that antibodies can be stored in the lymphocytes and released under the influence of the cortical hormones. The diminution in circulating lymphocyte counts, which has been reported by Chase, White and Dougherty to coincide with an increased antibody titre, was certainly obtained in the experiments under discussion but no concomitant rise in antibody levels was noted. This discrepancy between the two sets of experiments might be explained in at least three ways. The method of antibody titration selected by the present authors is essentially

that used by Hartley and Smith (1935) for the international standardisation of staphylococcal antitoxin. It involves the use of a quantity of toxin some 50 to 100 times greater than that used by Chase, White and Dougherty. Recognising that the method used by these workers might be sensitive to smaller changes in antibody titre, the sera obtained in one of the above experiments was titrated at different toxin levels. No difference in the relative levels of antitoxin could be detected, whether the titration was made against 1, 1/5 or 1/50 L.H. unit. Difference of technique therefore cannot, in this instance, explain the difference in results. Another reason for the lack of effective change in antibody titres may be that hyperimmune subjects were not used. This idea is suggested by the findings of Eisen *et al.* They noted that the animals which did show rises in antibody nitrogen were those which had previously shown high titres after immunization. The course of staphylococcal toxoid given to the subjects of the present experiments was designed for routine therapeutic use and consequently is not comparable to the antigen stimulus usually given to experimental animals. While it is possible to reach levels around 40 PIUs by repeated courses, this was not attempted. Possibly antibodies are only stored in lymphocytes when larger quantities of antibody have been manufactured and these conditions were not attained in the above experiments. Finally, it cannot be assumed that a fall in circulating lymphocyte counts necessarily reflects a destruction of these cells throughout the body. The rapidity with which the counts return to normal would, if this were so, indicate an amazing capacity of the lymphoid tissue to produce these cells. One would expect to find large numbers of very immature cells appearing in the circulation, a phenomenon which was not noticed while making the counts.

SUMMARY

Circulating antibody levels in normal human subjects were not increased following the administration of large quantities (40–400 mgs.) of adrenocorticotrophic hormone. The findings of other workers regarding blood eosinophil and lymphocyte counts have, however, been confirmed. Both types of cells showed diminished counts, the fall in eosinophils being more marked and of longer duration the larger the amount of hormone injected.

There was a rapid fall in the packed cell volume following the higher doses of corticotrophin which was maintained for many hours after the cell counts returned to normal. The increase in plasma volume was not accompanied by a lowered concentration of serum proteins.

ACKNOWLEDGMENTS

We wish to thank Professor J. S. L. Browne at whose suggestion these experiments were initiated and Professor E. G. D. Murray,

both of whom have given us much help throughout the course of the work. Our thanks are also due to the experimental subjects for their very patient co-operation, and to Armour Laboratories Inc. for the gift of the hormone preparations used in this work. One of us (P.H.H.) is indebted to the Banting Research Foundation for a grant.

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ADRENO-CORTICAL CHANGES IN SYRIAN HAMSTERS FOLLOWING GONADECTOMY

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IN THE course of gonadectomy studies with Syrian hamsters, changes in the suprarenal cortex were observed which may be of interest to other investigators. At the end of an experiment to test the effect of gonadectomy on dental caries in hamsters, autopsies were performed on all animals. A detailed account of the experimental procedures and other findings are available in previous reports (Keyes, 1946, 1948).

The weights of the suprarenal bodies are reported in Table 1. These data are in agreement with previous observations of Peczenik

TABLE 1. LITTER DISTRIBUTION OF ABSOLUTE AND RELATIVE SUPRARENAL WEIGHTS. VALUES IN MILLIGRAMS

	Control Males		Castrated Males		Control Females		Spayed Females	
Litter	Absolute Weight	mg/100 gm of body wt.	Absolute Weight	mg/100 gm of body wt.	Absolute Weight	mg/100 gm of body wt.	Absolute Weight	mg/100 gm of body wt.
B	16	16.7	15*	8.8	13	7.5	12	6.7
C	26	23.8	16	10.0	11	6.8	15	10.0
D	25	24.8	16	10.1	10	6.8	13	8.1
E	20	18.3	18	11.0	11	7.4	17	10.6
G	26	24.0	21	12.5	10	5.8	18	11.0
H	20	20.8	17	10.7	15*	9.6	15*	9.4
I	16	20.5	16	12.0	12*	7.5	12	9.2
K	19	19.2	16	12.2			13*	9.5
L			17	11.2	15	9.7	16	11.3
M	21*	22.2	20*	12.8	16	13.2	15	10.7
M	21	20.2			15	12.2		
Mean Wt.	21	20.9	17	11.2	13	8.7	15	9.7

* Organ was slightly damaged in dissection. Values are not included in average weights, either absolute or relative.

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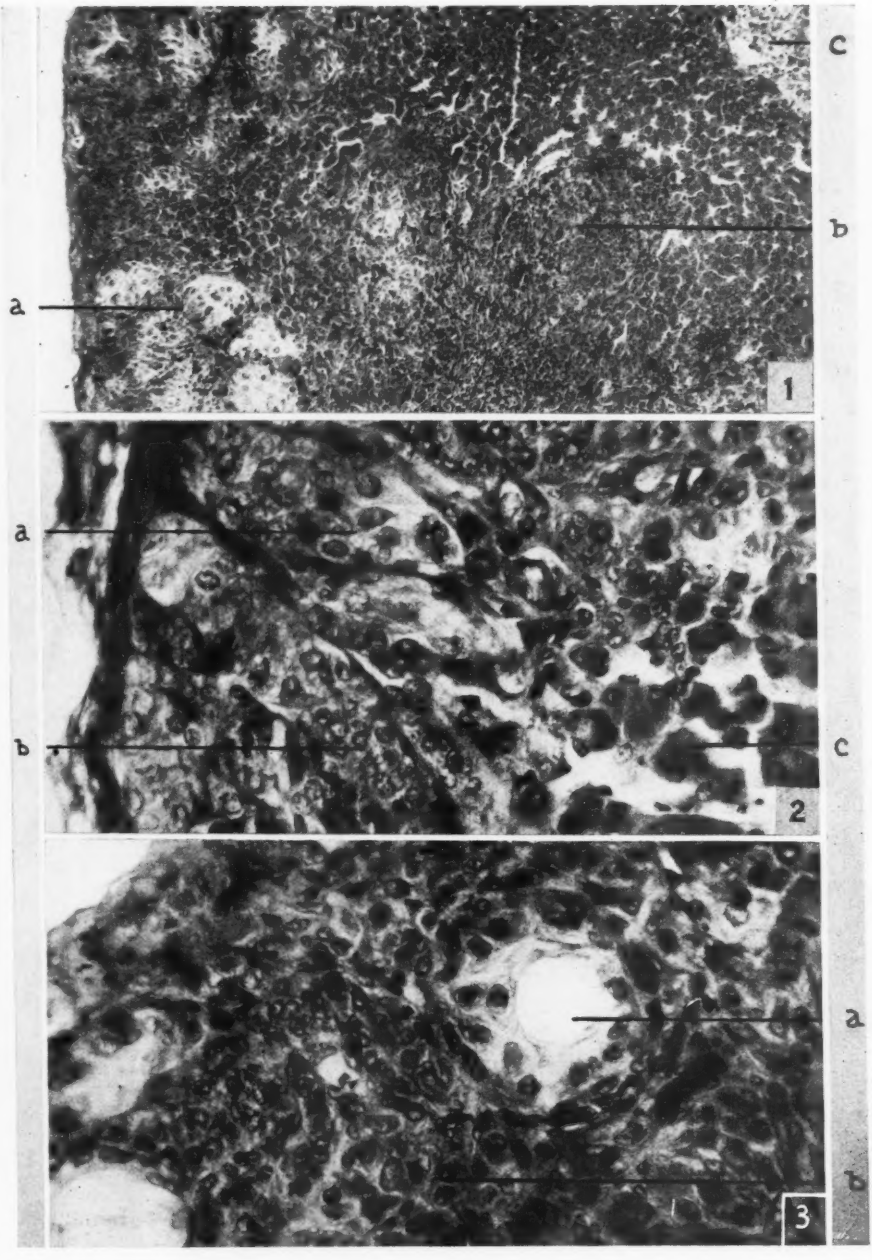
Sections of suprarenal cortex of gonadectomized hamsters. Zenker fixation; hematoxylin and eosin.

FIG. 1. a) Groups of enlarged cells in zona glomerulosa. b) Invasion of zona fasciculata by spindle-shaped cells. c) Medulla. (×100)

FIG. 2. a) Hypertrophied subcapsular cells in zona glomerulosa. b) Densely packed cells with indistinct cytoplasm and nuclei containing coarse chromatin material. c) Normal fasisculata cells. (×430)

FIG. 3. a) Follicle-like arrangement of large vesicular cells. b) Densely aggregated cells some of which appear to assume a spindle shape. (×430)


c
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(1944) that the suprarenals of male hamsters are absolutely and relatively larger than those of females; and castration of male hamsters is associated with a decrease in suprarenal size on both an absolute and relative weight basis. Peczenik (1944) reports that spaying is associated with increased adrenal weight in females. Our data also show this tendency. Koneff et al. (1946) also report that the adrenals of normal male hamsters are heavier than those of normal females.

The surface of the suprarenal bodies of all gonadectomized animals, male and female, were characterized by the presence of small whitish pin-point spots. None of the control animals showed these changes. Histological sections of normal and abnormal glands were prepared and stained with hemotoxylin and eosin. Serial sections were not made.

Figures 1, 2, and 3 show the typical castration changes observed in the hamster adrenal. There was a subcapsular proliferation of cells in the outer part of the cortex which occurred largely at the expense of the zona fasciculata. In the sections studied, marked displacement of the medulla was not a common finding. Mitotic figures were not frequently seen, and no malignant tendencies were observed.

In some areas changes appeared to be an hypertrophy of the zona glomerulosa. Cell bodies were large and boundaries faintly outlined. The cells were arranged in clusters and occasionally in follicle-like patterns. Nuclei appeared large and ovoid and contained scattered chromatin material. The cytoplasm stained very lightly with eosin.

In other areas smaller subcapsular cells were found in localized aggregations so densely packed that cell boundaries were indistinguishable. Nuclei were large and ovoid and contained coarse granular material. The cytoplasm was scanty, granular, and lightly stained with eosin. In certain areas these cells appeared to be increasing in size and tended to resemble the hypertrophied cells of the zona glomerulosa described above. These smaller cells were found in groups surrounded by spindle-shaped cells. In other areas spindle-shaped cells appeared to be infiltrating between the fascicular columns.

Adreno-cortical changes have been reported in gonadectomized rats, mice, and guinea pigs. Wooley and coworkers have described nodular hyperplasia of the suprarenal cortex in mice ovariectomized at birth (1939) and comparable changes in castrated male mice (1941). Hall (1940) describes a narrowing of the zona glomerulosa and other histological changes in the adrenal cortex of gonadectomized male and female rats. Fekete et al. (1941) report the histological changes in cortex of ovariectomized dba mice and classified these changes as nodular hyperplasia. Their description and photographs in many respects resemble the findings in hamsters. Tumors of the adrenal cortex following gonadectomy have been reported in mice, rats, and guinea pigs by a number of investigators; Gardner (1941), Heiman (1944), Spiegel (1939), Woolley et al. (1945 a, b).

In a subsequent study 25 mg pellets of testosterone propionate were implanted at the time of operation into 5 castrated male and 10 spayed female hamsters 22 to 38 days old. Control groups included 12 unoperated and untreated males and an equal number of females; also 10 castrated males and six spayed females. After 137 days on an experimental regimen, the animals were killed. Changes similar to those previously mentioned were observed in the suprarenals of the 16 gonadectomized animals which received no testosterone. No gross changes were detected in the suprarenals of gonadectomized males and females treated with testosterone. A few unoperated control animals had suspicious white spots on the surface of the adrenal bodies. These animals also had comparatively small testes and ovaries. The effect of seasonal variation on the control animals is not known, as this experiment was conducted during the winter months.

Histological sections were made of some of the suprarenals in this experiment. Serial sections were not prepared. Cytological changes were found in the cortex of all gonadectomized male and female animals and were similar to those described. Testosterone propionate prevented the change in gonadectomized males and females; one testosterone treated male, however, showed questionable changes. Slight changes were also noted in the cortex of some of the control animals.

SUMMARY

Cytological changes in the adrenal cortex of gonadectomized male and female hamsters resembled the description of nodular hyperplasia reported by other workers. Testosterone propionate in the form of 25 mg. pellets prevented the adreno-cortical changes.

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QUANTITATIVE INTERFERENCE WITH ESTROGEN-INDUCED TISSUE GROWTH BY FOLIC ACID ANTAGONISTS[1]

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WE HAVE previously reported that the folic-acid deficient chick exhibits only a very limited response to even excessively high doses of administered estrogen (Hertz, 1945). A similar impairment in estrogen response has been observed in the folic-acid-deficient monkey (Hertz, 1948). Moreover, the degree of response to injected estrogen as evidenced by increase in weight of the genital tract in the chick was shown to be quantitatively related to the amount of folic acid ingested. These observations have been confirmed by Kline and Dorfman (1948).

Preliminary data have been previously presented indicating that the ingestion of a chemically unidentified, crude folic acid antagonist incorporated into a normal stock diet leads to a marked reduction in genital tract response to diethylstilbestrol in chicks (Hertz, 1948). Franklin et al. (1948) have reported similar observations.

The present report is concerned with further quantitative studies on the inhibition of estrogen-induced tissue growth in the genital tract of both the chick and rat by means of certain chemically identified folic acid antagonists.

METHODS AND MATERIALS

(A) Chicks: New Hampshire Red chicks of the same flock were used throughout.[2] They were received the day after hatching and were maintained in electrically heated brooders. No food was given but tap water was provided ad libitum. On each of the first three days in the laboratory the chicks were injected subcutaneously with from 0.2 cc. to 1.0 cc. of an aqueous solution or suspension of the folic acid antagonist to be tested. In experiments designed to show the reversibility of the observed inhibition, the chicks also received subcutaneously each day 5 mg. of folic acid dissolved in 1 cc. of .05/N NaOH. The folic acid was always injected one hour before the administration of the antagonist. On each of the last two days of the test, 1 mg. of diethylstilbestrol dissolved in 0.2 cc. of corn oil was injected subcutaneously. Twenty-four hours after the last injection the animals were

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[1] Read before the annual meeting of the Association for the Study of Internal Secretions; June 19, 1948, Chicago, Ill.

[2] Obtained from Hall Bros. Hatchery, Wallingford, Conn.

autopsied. The body weight was recorded and the genital tract dissected out and weighed. Control groups of animals receiving diethylstilbestrol alone and those receiving no treatment were autopsied at the same time.

(B) Rats: Twenty-one day old weanling female rats of the Wistar Strain were ovariectomized and given a pulverized stock ration[3] supplemented with 2 per cent dried liver ad libitum. After a rest period of from 3 to 5 days each rat was given the test dose of folic acid antagonist subcutaneously in 0.2 cc. to 1 cc. of an aqueous solution or suspension on each of three consecutive days. On the second and third day of treatment the rats also received 10 micrograms of estradiol in 0.5 cc. distilled H_2O subcutaneously. In the reversal experiments, the folic acid was administered subcutaneously in 1 cc. of .05/N NaOH at the rate of 5 mg. per day for 4 consecutive days, starting the day preceding treatment with the antagonist. At autopsy, 24 hours after the last injection, the uteri were dissected out, freed of distending fluid, and weighed to the nearest milligram.

The folic acid employed was crystalline pteroylglutamic acid. The antagonists investigated included:[4]

(I) 4-aminopteroylglutamic acid
(II) 4-aminopteroylaspartic acid
(III) 4-amino-N^{10}-methyl pteroylglutamic acid
(IV) 2,4-diamino-6,7-dimethyl pteridine
(V) 2-amino-4-hydroxy-6,7-di(p-amino-phenyl) pteridine
(VI) 2,4-diamino-6,7-diphenyl pteridine
(VII) 4-desoxypteroylglutamic acid
(VIII) 2-amino-4-hydroxy-6,7-diphenyl pteridine

RESULTS AND DISCUSSION

Most of the detailed quantitative studies were done with 4-aminopteroylglutamic acid (I).

Figure 1 presents the data from a representative experimental chick series. It will be seen that doses of from 4 to 16 micrograms of 4-aminopteroylglutamic acid (I) effect progressive decrements in oviduct weight. This impairment in oviduct response is readily reversed by administration of an excess of folic acid.

Similar observations in the rat are summarized from 3 successive experiments in Fig. II. As in the chick, the significant features are (a) the distinct quantitative relationship between the inhibition obtained and the dose of antagonist administered and (b) the nearly complete reversibility of the inhibition by high doses of folic acid.

The observations made with the other antagonists on the chick are summarized in Table 1. It will be seen that 4-amino-N^{10}-methyl folic acid and 4-aminopteroylaspartic acid also exert an inhibitory effect which is reversible by folic acid in the former. A limited inhibitory effect is also observed with 4-desoxypteroyl-glutamic acid. The

[3] Purina Chow.

[4] (I) (II) (III) and crystalline folic acid kindly supplied by the Lederle Laboratories, Pearl River, New York. (IV) (V) (VII) kindly supplied by Dr. R. A. Brown, Parke Davis & Co., Detroit, Michigan. (VIII) and (VI) kindly furnished by Dr. C. K. Cain, Cornell University, Ithaca, New York.

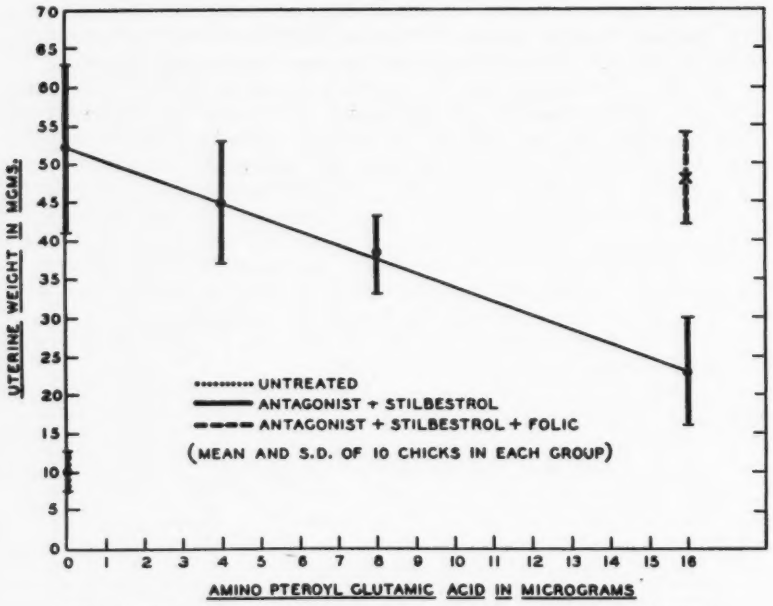

FIG. 1. Effect of folic antagonist on chick oviduct response to stilbestrol.

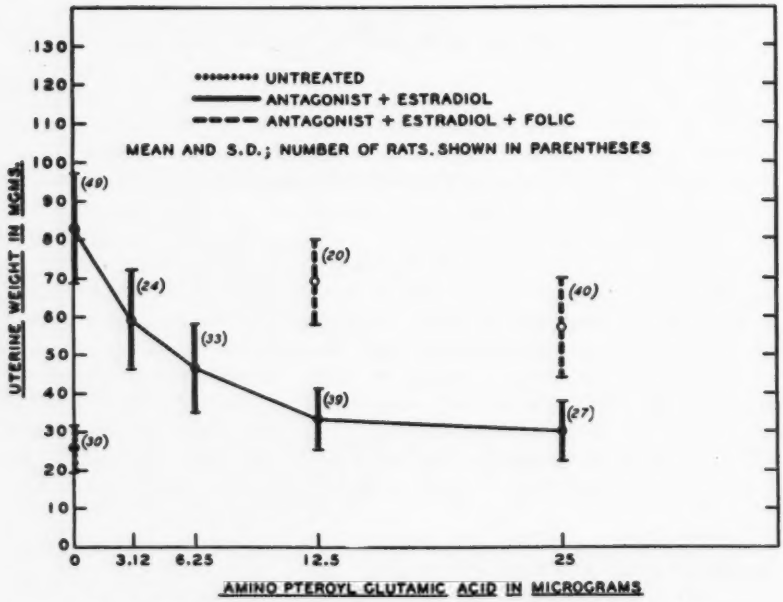

FIG. 2. Effect of folic antagonist on rat uterine response of estradiol.

pteridine compounds proved essentially ineffective at the moderate doses employed. These negative data serve to indicate the specificity of the inhibition obtained with the more active compounds.

The marked dependence of a specific tissue growth response upon a dietary trace factor is a phenomenon of both practical and theoreti-

TABLE 1. EFFECT OF FOLIC ACID ANTAGONISTS ON ESTROGEN RESPONSE IN CHICK OVIDUCT[1]

Antagonist	Daily Dose mg.	Oviduct Weight mg.
4-amino-N^{10}-methyl pteroylglutamic acid	0.2	23 ±5
	1.0	23 ±3
	0.2[a]	57 ±8
	0.0	51 ±6
4-aminopteroylaspartic acid	0.2	48 ±5
	0.4	44 ±5
	0.8	33 ±9
	2.0	29 ±6
	3.0	22 ±7
	4.0	20 ±3
	0.0	54 ±8
4-desoxypteroylglutamic acid	1.0	50 ±7
	5.0	55 ±6
	25.0	47 ±6
	50.0	23 ±4
	0.0	59 ±5
2,4-diamino-6,7-dimethyl pteridine	1.0	48 ±5
	5.0	58 ±12
2-amino-4-hydroxy-6,7-diphenyl pteridine	1.0	50 ±4
	5.0	53 ±5
	9.0	47 ±4
2-amino-4-hydroxy-6,7-di(p-aminophenyl) pteridine	1.0	48 ±6
	5.0	62 ±11
2,4-diamino-6,7-diphenyl pteridine	1.0	52 ±4
	5.0	51 ±8
	9.0	52 ±9

[1] All animals treated as described in text; 10 chicks in each group.
[a] Also given 5 mg. folic acid daily.

cal interest. Further study of this and related effects may serve to elucidate the metabolic mechanisms involved in this type of hormonal response. In the case of folic acid there is not yet decisive information as to the precise metabolic role of this vital substance in cellular metabolism. There is some indication that the vitamin is concerned with the metabolism of thymine and other pyrimidines and purines (Stokes, 1944). However, when the essential function of folic acid becomes clarified, we may also gain further knowledge of some of the mechanisms involved in estrogen-induced tissue growth.

The quantitative character of the inhibition obtained in both the

chick and the rat suggests an intimate and dynamic relationship between the hormone and the vitamin. It is noteworthy that this relationship applies to estrogens of both the stilbene and steroid type and can be demonstrated in both bird and mammal.

The available data do not warrant the conclusion that the interaction between folic acid and its antagonists in the quantitative determination of estrogen-induced tissue growth may be considered a strictly competitive relationship. Before such a conclusion may be reached there will be required data indicating the minimal amount of folic acid needed to reverse the inhibition produced by variable amounts of antagonist. The data at hand simply demonstrate that the expected level of tissue growth may be restored by an excess of folic acid.

It should be emphasized that the growth inhibition effected by the folic acid antagonists occurs in a tissue which is under the influence of a highly potent growth stimulus administered at enormous dose levels. Further study of the quantitative ratio between hormone dose and the quantity of antagonist required for maximum inhibition may serve to further characterize the vitamin-hormone relationship.

The interference with the action of a hormone by an anti-vitamin has certain implications for therapy in those conditions in which it may prove desirable to reduce the biological effectiveness of endogenous hormones, as in the case of prostatic carcinoma (Huggins et al. 1941); and cancer of the breast (Boyd, 1900). The present observations may serve as a basis for further exploration in this field.

SUMMARY

Quantitative inhibition of estrogen-induced tissue growth in the female genital tract by a number of folic acid antagonists is demonstrated in the stilbestrol-treated chick and in the estradiol-treated ovariectomized rat. This inhibition is reversed by the administration of folic acid.

The significance of quantitative interference with hormonally induced tissue growth by an anti-vitamin is discussed.

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THE ELEVATION OF PLASMA RIBOFLAVIN IN ESTROGEN TREATED FEMALE CHICKS

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A MARKED increase in the fluorimetrically determined riboflavin content of the serum of the immature female domestic fowl undergoing treatment with estradiol dipropionate was reported by Comman and Bolton (1946), and Common, Rutledge and Bolton (1947).

We have further investigated this phenomenon as a part of a more general study of the role of essential nutrients in hormonal stimulation of tissue growth. An increase in plasma riboflavin was observed in female chicks treated with diethylstilbestrol, the riboflavin being determined both fluorimetrically and microbiologically. In addition, we have attempted to determine the source of the elevated plasma riboflavin by analyses of muscle and liver of chicks maintained on a complete and on a riboflavin deficient diet.

It was also observed that the blood nicotinic acid content, in marked contrast with the riboflavin content, is not materially altered by estrogen administration.

MATERIALS AND METHODS

New Hampshire Red female chicks from the same flock were used throughout.[1] These chicks were received the day after hatching and were placed immediately either on a stock diet,[2] or on a synthetic diet of known riboflavin content (Franklin *et al.*, 1947).

Estrogen treatment consisted of the subcutaneous administration of 1 mg. diethylstilbestrol in 0.2 cc. corn oil daily for 5 days. Control chicks received equivalent amounts of corn oil alone. Treatment was begun when the chicks were 15 to 20 days of age. Autopsy was performed 24 hours after the last injection in all instances, except in those chicks described in Table 2, which were retained for the indicated intervals after injection in order to determine the rate of decline in riboflavin after cessation of treatment.

Blood and tissue samples taken from individual birds were prepared for assay according to Loy (1947). For the microbiological assays for riboflavin and nicotinic acid, Lactobacillus casei was grown on the medium described by Teply and Elvehjem (1945) and the method of Snell and Strong (1939) was employed.

The fluorimetric determinations were made by the official method of the

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[1] Obtained from Hall Bros. Hatchery, Wallingford, Conn.

[2] Purina Startena.

A.O.A.C. (1945), except that the addition of 3 drops of concentrated NaOH was employed for the development of the blank, this procedure having proven adequate for the complete inactivation of the riboflavin present (Robertson and Kahler, 1942).

RESULTS AND DISCUSSION

A marked elevation in plasma riboflavin following estrogen administration is seen in both normal and riboflavin deficient birds, although the rise is not as great in the deficient animal. Nevertheless, the liver and muscle content of riboflavin remained unaltered in both

TABLE 1. EFFECT OF DIETHYLSTILBESTROL ON RIBOFLAVIN CONTENT OF CHICK LIVER, PLASMA, AND MUSCLE*

Diet	Stilbestrol Treated	No. of Chicks	Assay Method[1]	Plasma	Liver	Muscle
Stock	+	24	M	1.21 ± .45	14.8 ± 1.2	2.2 ± .1
			F	.47 ± .20	15.5 ± 2.7	2.0 ± .3
	−	21	M	0.34 ± .08	13.9 ± 1.3	2.6 ± .1
			F	.07 ± .04	14.4 ± 2.1	2.0 ± .2
Riboflavin Deficient	+	12	M	0.61 ± .19	7.8 ± 0.5	2.5 ± .4
			F	.09 ± .03	7.8 ± 1.4	2.1 ± .4
	−	18	M	0.32 ± .08	8.7 ± 2.1	2.4 ± .3
			F	.06 ± .08	9.0 ± 1.8	1.9 ± .2

* All values are expressed as micrograms of riboflavin per gm. of fresh tissue or cc. of fresh plasma.
[1] F = fluorimetric; M = microbiological.

normal and deficient chicks receiving estrogen. The increase in plasma riboflavin can be induced by estrogen administration in the deficient bird even though the liver content of riboflavin is reduced to about half the normal level. The muscle content is unaffected either by the riboflavin deficiency or by estrogen treatment. The data therefore indicate that the serum elevation is not necessarily accompanied by a marked tissue depletion and that this increase in plasma level does not require full tissue saturation.

The maintenance of an elevated plasma riboflavin level after cessation of estrogen injection continues for less than six days, (Table 2). These data suggest that the continued mobilization of the vitamin requires a relatively high hormone level.

The nicotinic acid content of the blood was determined largely for comparison with the riboflavin content. The data in Table 3 indicate that the blood content of nicotinic acid is not materially altered by estrogen administration. This observation serves to emphasize the significance and specificity of the riboflavin effect.

It will be noted (Table 1) that there is excellent agreement in the values obtained by fluorimetric and microbiological assay in the case of liver and muscle. However, the serum assays show a higher value for riboflavin by the microbiological method than by fluorimetric de-

TABLE 2. BLOOD RIBOFLAVIN AFTER DISCONTINUATION OF ESTROGEN

No. of Days*	No. of Chicks	Assay[1]
1	6	0.79 (.21 –1.7)
6	4	0.23 (0.17–0.27)
11	4	0.18 (0.15–0.21)
15	4	0.22 (0.12–0.27)
20	3	0.11 (0.08–0.13)
25	4	0.16 (.14– .18)
—**	4	0.15 (0.12–0.18)
—***	4	0.12 (.08– .15)

* Number of days after last stilbestrol injection.
** Untreated Controls killed at beginning of experiment.
*** Untreated Controls killed at end of experiment.
[1] Microbiologically determined riboflavin in micrograms per cc. fresh blood; range in parentheses.

TABLE 3. EFFECT OF ESTROGEN ON BLOOD CONTENT OF NICOTINIC ACID

Sample	Stilbestrol	Assay[1]
A	+	8.0
	–	10.0
B	+	8.0
	–	10.5
C	+	10.9
	–	12.4
D	+	9.3
	–	12.7

[1] Expressed as micrograms of nicotinic acid per cc. fresh blood.

termination. This discrepancy is probably attributable to the presence in chick blood of non-specific lipid materials which stimulate the growth of L. casei, (Strong and Carpenter, 1942). It is pertinent that estrogen administration leads to a marked lipemia in the bird, (Fleischmann and Fried, 1945). Such a lipemia would presumably increase the blood content of the factors stimulating bacterial growth. Accordingly, further study of the hormonal factors involved in the

mobilization of these growth-promoting lipids and their potential effect upon the tissue growth response to estrogens in the female genital tract may prove fruitful. Moreover, our microbiological assays for riboflavin in plasma must be considered only as comparative rather than as absolute values.

SUMMARY

Earlier reports of a marked increase in plasma riboflavin in the chick after estrogen administration have been confirmed. This elevation in plasma riboflavin titre was also observed in riboflavin deficient chicks. The muscle and liver content of riboflavin was not altered by estrogen administration in either normal or riboflavin deficient chicks. In contrast to the riboflavin effect, the nicotinic acid content of the blood remained unaltered after estrogen treatment. The blood riboflavin levels returned to normal within six days following cessation of estrogen injections.

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NOTES AND COMMENTS

INTERPRETATION OF EXPERIMENTAL RESULTS OBTAINED WITH DIBENAMINE[1]

Demonstration of the highly effective and specific adrenergic blocking action of Dibenamine (N,N-dibenzyl-β-chloroethylamine) (Nickerson and Goodman, 1945, 1947, 1948; Nickerson and Nomaguchi, 1948) suggested its use as a tool in determining the role of excitatory adrenergic stimuli (epinephrine or sympathetic nerve impulses) in a variety of physiological processes. Among the papers reporting the use of Dibenamine in this capacity are those of Sawyer *et al.* (1947, 1948) on the prevention of post-copulatory ovulation in the rabbit by Dibenamine, and that of Rockwell (1948) on the therapeutic effect of Dibenamine in certain psychoses. In both cases, the conclusion was reached that blockade of adrenergic stimuli was causally related to the effects observed after the administration of Dibenamine. A careful analysis of the pharmacology of this drug indicates that alternative explanations must be considered.

Although the adrenergic blocking action of Dibenamine (and related β-haloalkylamines) usually overshadows all other actions of these compounds, it must be kept in mind that no known drug is absolutely specific. The following points are of particular importance in evaluating the results of experiments employing Dibenamine.

1. Dibenamine has a direct excitant action on the central nervous system. This effect appears to be independent of the adrenergic blocking action. It occurs earlier and is of much shorter duration than the blockade. Central nervous system excitation is particularly prominent after rapid intravenous administration of the drug and under these circumstances may be the most prominent action.

2. Dibenamine has been shown to block primarily the excitant effects of adrenergic stimuli on smooth muscle and exocrine gland cells. It has not been shown to block any adrenergic metabolic effects or the actions of adrenergic stimulation on the central nervous system, such as hyperventilation, analepsis, etc.

3. In contrast to the direct central stimulating action of Dibenamine, the adrenergic blocking action develops slowly. This action appears earlier when large doses of the drug are employed, but present results indicate that maximum blockade does not occur until at least 1½ hours after intravenous administration.

All these points apply to an interpretation of the work of Sawyer and associates. The rapid injection and short time-intervals involved in their experiments at once suggest the possibility that the direct effect of Dibenamine on the central nervous system rather than its adrenergic blocking

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[1] Dibenamine and 2-dibenzylaminoethanol were kindly supplied by Dr. William Gump of Givaudan-Delawanna, Inc.

action may have been the critical factor in preventing ovulation. (The injections in their experiments were regularly followed by signs of extreme central nervous system stimulation.) In the work of Rockwell, cerebral vascular changes as well as direct central nervous system stimulation must be considered.

Fortunately, the above difficulties of interpretation can be largely overcome by control experiments employing 2-dibenzylaminoethanol. This compound is the hydrolysis product of Dibenamine in which the β-chlorine is replaced by an hydroxyl group. It has no demonstrable adrenergic blocking or local necrotizing action, but retains most of the other pharmacological properties of Dibenamine. Its direct action as a central nervous system stimulant in comparison with that of Dibenamine is shown in Table 1. The slightly

TABLE 1. COMPARISON OF CENTRAL NERVOUS SYSTEM STIMULATION PRODUCED BY DIBENAMINE AND 2-DIBENZYLAMINOETHANOL

Dose[1] mg./kg.	No. of Mice	Convulsions %	Ave. Duration of Convulsions[2] min.	Mortality[3] %
	DIBENAMINE			
25	10	80	0.29 ±0.07	0
35	10	100	2.8 ±0.60	0
50	10	100	7.9 ±1.7	40
	2-DIBENZYLAMINOETHANOL			
18	10	60	0.66 ±0.25	0
25	15	100	2.2 ±0.46	40
35	15	100	4.0 ±0.53	53

[1] All injections had a volume of 5–10 ml./kg. and were made in 5 seconds.
[2] In animals which survived.
[3] All deaths were due to asphyxia during convulsions.

greater potency of 2-dibenzylaminoethanol and its somewhat greater ratio of lethality to duration of convulsions are probably attributable to its greater aqueous solubility. The compound also produces an increased respiratory exchange and other signs of central nervous system stimulation.

It may be hoped that the employment of 2-dibenzylaminoethanol as a control substance in experiments involving the adrenergic blocking action of Dibenamine will materially clarify interpretations of the results obtained.

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MALE MATING BEHAVIOUR SHOWN BY FEMALE RATS TREATED WITH ENORMOUS DOSES OF ESTRONE

Stone 1924 and Beach 1938 reported that in male rats, which were vigorous copulators, a tendency to develop certain elements of female mating behaviour could be observed. Later, Beach (1941) demonstrated that large doses of testosterone-propionate elicit female mating behavior in male rats, especially if these animals are excited by a previous copulation which was interrupted before ejaculation.

We have shown (Engel, 1942) that similar effects can be produced in male mice by testosterone propionate as well as by estradiol benzoate or by yohimbine. We affirmed Beach's theory that in those cases the reactions are rather due to a sexual (ambivalent) excitation of the nervous system, which is capable of producing the mating pattern of either sex. Recently we have extended this theory by observations in female rats, where also sex hormones elicit an apparently homosexual or lesbic reaction. Beach (1942) had shown that testosterone may produce male mating behaviour in spayed female rats.

Two groups of rats, one of four adult female rats, which had been spayed one year ago being then very young, virginal animals, and a second group of three adult normal female rats aged one year, received each one a deposit of 10 milligrams (100,000 I.U.) of estrone in form of microcrystals by intramuscular injection into the right hind leg.

From the second day and during a long period following, the animals of both groups were in perfect estrus. In order to determine the permanence of this state, vaginal smears were taken. From the tenth day we observed that after taking the vaginal smears (which was done with a small piece of cotton fixed on a very fine pincette), all animals were in a state of sexual hyperexcitation. It should be mentioned that the spayed animals had never had any contact with a male, and the normal females had not had such intercourse for some months. In the present experiment we observed that the excited animals began to chase each other, in the same manner a male rat would do, licking the genital region and afterwards trying to mount.

Normal, or spayed females, excited only by friction of the vagina, never showed a similar reaction in presence of other female rats, whilst each of the rats treated with 100,000 I.U. of estrone reacted as noted above. There was no difference between spayed and normal female rats; the animals not only attacked rats in permanent estrus, but also proestric, or metestric females, as well as spayed rats. But, as there was no reaction on the side of the attacked animals, the pursuers quickly ceased to be interested in them.

This indicates a certain difference between the behaviour of the normal male, which will attack only estrous females, and treated animals. It agrees very well with Beach's observation that young male rats, made "hyperexcitable" by testosterone-propionate, displayed a tendency to mate with non-receptive female rats, male rats, and even guinea pigs.

Once more we come to the conclusion that a very strong hormonal hyperexcitation elicits the nervous reactions in sexual behavior to attack whatever object; but this is rather to be considered as a general hypersexuality than as a transformation into a homosexual attitude.

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SUMMARY

Female rats, entire as well as spayed, injected with 10 mgms. of estrone in microcrystals, excited by friction in taking vaginal smears, attack other females of their species, chasing and even mounting them, showing male mating behaviour.

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